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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12Q 1/68, C12P 19/34	A1	(11) International Publication Number: WO 99/43854 (43) International Publication Date: 2 September 1999 (02.09.99)
(21) International Application Number: PCT/US99/04049 (22) International Filing Date: 25 February 1999 (25.02.99) (30) Priority Data: 60/076,161 26 February 1998 (26.02.98) US (71) Applicant: THE OHIO STATE RESEARCH FOUNDATION [US/US]; 1960 Kenny Road, Columbus, OH 43210 (US). (72) Inventors: LAMBOWITZ, Allen, M.; 4204 Benedict Lane, Austin, TX 78741 (US). ZIMMERLY, Steven; 1902 Westmount Road N.W., Calgary, Alberta AB T2N (CA). GUO, Huatao; 1845 Burton Drive #156, Austin, TX 78741 (US). MOHR, George; 4514 Avenue C, Austin, TX 78751 (US). BEALL, Clifford, James; 58 King Avenue, Columbus, OH 43202 (US). (74) Agent: DOCHERTY, Pamela, A.; Calfee, Halter & Griswold LLP, Suite 1400, 800 Superior Avenue, Cleveland, OH 44114 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHODS FOR CLEAVING SINGLE-STRANDED AND DOUBLE-STRANDED DNA SUBSTRATES WITH NUCLEOTIDE INTEGRASE (57) Abstract Methods, employing a nucleotide integrase, for cleaving single-stranded RNA substrates, single-stranded DNA substrates, and double-stranded DNA substrates at specific sites and for inserting a nucleic acid molecule into the cleaved substrate are provided. One method uses a nucleotide integrase to cleave one strand of a double-stranded DNA substrate. The method comprises the steps of: providing an isolated nucleotide integrase comprising a group II intron RNA having two hybridizing sequences for hybridizing with two intron RNA binding sequences on the top strand of the DNA substrate, and a group II-intron encoded protein which binds to a first sequence element of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate to permit the nucleotide integrase to cleave the top strand of the DNA substrate and to insert the group II intron RNA into the cleavage site. The method of cleaving both strands of a double-stranded DNA substrate comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two hybridizing sequences for hybridizing with two intron RNA binding sequences on one strand of the substrate, and a group II-intron encoded protein that interacts with a first sequence element and a second sequence element in the recognition site of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate such that the nucleotide integrase cleaves both strands of the DNA substrate and inserts the group II intron RNA into the cleavage site of the one strand. The method for cleaving a single-stranded nucleic acid substrate comprises the steps of: providing a nucleotide integrase having two hybridizing sequences for hybridizing with two intron RNA-binding sequences on the single-stranded substrate, and a group II-intron encoded protein; and reacting the nucleotide integrase with the single stranded nucleic acid substrate to allow the nucleotide integrase to cleave the substrate and to attach the group II intron RNA molecule thereto.		

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METHODS FOR CLEAVING SINGLE-STRANDED AND DOUBLE-STRANDED DNA SUBSTRATES WITH NUCLEOTIDE INTEGRASE

The present invention was made with support from National Institutes of Health Grant NO. GM37949. The United States Government has certain rights in the invention.

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BACKGROUND

In recent years, a number of methods have been developed for manipulating DNA. Some of these methods employ biomolecules to cut or cleave DNA, which in some instances renders the substrate DNA nonfunctional. Other methods employ biomolecules to facilitate insertion of new pieces of nucleic acid into the cleavage site of the DNA substrate. The insertion of new segments of nucleic acid into the cleavage sites of the DNA substrate changes the characteristics of the RNA or protein molecules encoded by the substrate DNA molecules. Accordingly, the biomolecules which catalyze the cleavage of DNA substrates or the insertion of new nucleic acid molecules into the DNA substrates are useful tools for genetic engineering, for analytical studies and for diagnostic studies. One such molecule used for cleaving DNA substrates is the restriction endonuclease.

Restriction endonucleases are enzymatic proteins that cleave double-stranded DNA. Such endonucleases recognize specific nucleotide sequences in double-stranded DNA, and cleave both strands within or near the specific recognition site. Such specificity renders the restriction endonucleases important tools in the controlled fragmentation of double-stranded DNA. Restriction endonucleases are also useful analytical tools for determining whether certain sequences are present in substrate DNA and in genomic sequencing studies.

However, restriction endonucleases only cleave DNA substrates; they do not insert new nucleic acid molecules into the cleaved DNA substrate. Accordingly, another biomolecule is needed to insert new pieces of DNA or RNA into the double-stranded DNA.

Ribozymes are catalytic RNA molecules that cleave RNA and, in certain circumstances, that insert new pieces of RNA into the cleavage site of the RNA substrate. Unfortunately, ribozymes have not been particularly useful for cleaving single-stranded DNA substrates or double-stranded DNA substrates. Ribozymes cut single-stranded DNA only under extreme conditions of elevated temperatures and high concentrations of magnesium. Ribozymes can be used to cleave double-stranded DNA only after the DNA is denatured and separated into two pieces of single-stranded DNA. Moreover, ribozymes have limited use in systems containing ribonucleases.

Accordingly, it would be desirable to have methods which employ a new tool that is capable of cleaving double-stranded DNA molecules, single-stranded DNA molecules, and single-stranded RNA molecules at specific sites. Methods which employ a new biomolecule capable of cleaving RNA molecules, single-stranded DNA molecules and double-stranded DNA molecules at specific sites and simultaneously inserting a new nucleic acid molecule into the cleavage site are especially desirable.

SUMMARY OF THE INVENTION

The present invention provides new methods, employing a nucleotide integrase, for cleaving single-stranded RNA substrates, single-stranded DNA substrates, and double-stranded DNA substrates at specific sites and for inserting nucleic acid molecules into the cleaved substrate. The nucleotide integrase is a ribonucleoprotein particle comprising a group II intron RNA and a group II intron-encoded protein, which is bound to the group II intron RNA.

One method uses a nucleotide integrase to cleave one strand, hereinafter referred to as the "top strand" of a double-stranded DNA substrate. The method comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two hybridizing sequences, "EBS1" and "EBS2", that are capable of hybridizing with two intron RNA binding sequences, "IBS1" and "IBS2", respectively, on the top strand of the DNA substrate, and a group II intron-encoded protein which binds to a first sequence element of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate under conditions that permit the nucleotide integrase to cleave the top strand of the DNA substrate and to insert the group II intron RNA into the cleavage site. Preferably, the nucleotide immediately preceding the first nucleotide of the EBS1 sequence on the group II intron RNA, hereinafter referred to as the δ nucleotide is complementary to the nucleotide at +1 on the top strand of the substrate, hereinafter referred to as the δ' nucleotide.

As denoted herein, nucleotides that are located upstream of the cleavage site have a (-) position relative to the cleavage site, and nucleotides that are located downstream of the cleavage site have a (+) position relative to the cleavage site. Thus, in the above-described method, the cleavage site is located between nucleotides -1 and +1 on the top strand of the double-stranded DNA substrate. The IBS1 sequence and the IBS2 sequence lie in a region of the recognition site which extends from about position -1 to about position -14 relative to the cleavage site. As denoted herein, the first sequence element comprises from about 10 to about 12 pairs of nucleotides that lie upstream of IBS2 and IBS1, i.e from about position -12 relative to the cleavage site to about position -26 relative to the cleavage site. As denoted herein, the

second sequence element comprises from about 10 to about 12 pairs of nucleotides that lie downstream of the cleavage site, i.e., at positions +1 to about +12. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides and has substantial complementarity with the nucleotides at positions -1 to about -5 or about -7 on the top strand of the DNA substrate. The EBS2 sequence comprises from about 4 to 7 nucleotides and has substantial complementarity with the nucleotides at positions from about -6 to about -14 on the top strand of the DNA substrate.

The present invention also provides a method which employs a nucleotide integrase to cleave both strands of a double-stranded DNA substrate. The method comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two hybridizing sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA binding sequences, IBS1 and IBS2, on the top strand of the substrate, and a group II-intron encoded protein that is capable of binding to a first sequence element and to a second sequence element in the recognition site of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate such that the nucleotide integrase cleaves both strands of the DNA substrate and inserts the group II intron RNA into the cleavage site of the top strand. As denoted herein, the second sequence element comprises from about 10 to about 12 pairs of nucleotides that lie downstream of the cleavage site, i.e. from position +1 to about position +10, +11, or +12. Preferably, the δ nucleotide of the group II intron RNA is complementary to the δ' nucleotide on the top strand of the substrate.

Another method provided by the present invention employs a nucleotide integrase for cleaving a single stranded nucleic acid substrate and for inserting the group II intron RNA of the nucleotide integrase into the cleavage site. The method comprises the steps of: providing a nucleotide integrase having two hybridizing sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, on the single-stranded substrate, and a group II intron encoded protein; and reacting the nucleotide integrase with the single stranded nucleic acid substrate for a time and at a temperature sufficient to allow the nucleotide integrase to cleave the substrate and to attach the group II intron RNA molecule thereto. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides that have substantial complementarity with the nucleotides at positions -1 to about -5 or about -7 relative to the putative cleavage site. The EBS2 sequence comprises from about 4 to 7 nucleotides that have substantial complementarity with the nucleotides at positions from about -6 to about -13 relative to the putative cleavage site. Preferably, the δ nucleotide of the

group II intron RNA is complementary to the δ' nucleotide on the top strand of the substrate.

The present invention also relates to a method of determining whether a nucleic acid comprises a particular recognition site. The method comprises the steps of providing a nucleotide integrase capable of cleaving a nucleic acid comprising a particular recognition site; reacting the nucleotide integrase with the nucleic acid; and assaying for cleavage of the nucleic acid, wherein cleavage of the nucleic acid indicates that the nucleic acid comprises the recognition site.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram showing the interaction between the EBS sequences of a group II intron RNA of the second intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI2 intron" RNA and the IBS sequences of a DNA substrate. The cleavage site in the substrate is represented by an arrow.

Figure 2 is a diagram depicting the nucleotide sequence and the secondary structure of the aI2 intron RNA and the excised group II intron RNA of the first intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI1 intron" RNA.

Figure 3 is a chart depicting the wild-type sequence of the E2/E3 junction of the yeast mitochondrial *COX 1* gene and the position of the point mutations made in the wild-type sequence.

Figure 4 is a graph showing the relative extent of cleavage of the substrates having mutations in the nucleotides upstream of the cleavage site by a nucleotide integrase comprising a wild-type aI2 intron RNA and the protein encoded thereby.

Figure 5 is a graph showing the relative extent of cleavage of the substrates having mutations in the nucleotides downstream of the cleavage site by a nucleotide integrase comprising a wild-type aI2 intron RNA and the protein encoded by the aI2 intron RNA.

Figure 6 is a chart depicting the wild-type sequence of the E1/E2 junction of the yeast mitochondrial *COX 1* gene and the position of the point mutations made in the wild-type sequence.

Figure 7 is a graph showing the relative extent of cleavage of the substrates having mutations upstream of the cleavage site by a nucleotide integrase comprising a wild-type aI1 intron RNA the protein encoded thereby.

Figure 8 is a chart depicting the wild-type sequence of the E1/E2 of the *Lactococcus lactis* ltrB gene and the position of the point mutations made in this wild-type sequence.

Figure 9 is a graph showing the relative extent of cleavage of double-stranded DNA

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substrates by a nucleotide integrase comprising a wild-type group II intron RNA of the *Lactococcus lactis* *ltrB* gene, hereinafter referred to as the "Ll.*ltrB* intron" RNA, and the protein encoded thereby, hereinafter referred to as the *ltrA* protein.

Figure 10 shows the nucleotide sequence of the Ll.*ltrB* intron RNA plus portions of the exon sequences that flank the Ll.*ltrB* intron RNA.

Figure 11 is the amino acid sequence of the *ltrA* protein.

Figure 12 is a chart depicting the sequences of DNA substrates cleaved by nucleotide integrases comprising a wild-type or modified Ll.*ltrB* intron RNA and the *ltrA* protein.

Figure 13 is a graph showing the relative extent of cleavage of DNA substrates having multiple mutations by a nucleotide integrase comprising a wild-type Ll.*ltrB* intron RNA and the *ltrA* protein.

Figure 14 is a graph showing the relative extent of cleavage of the DNA substrates having a high G/C content in the first sequence element by a nucleotide integrase comprising a wild-type Ll.*ltrB* intron RNA and the *ltrA* protein.

Figure 15 is a graph showing the relative extent of cleavage of the substrates having an wild-type and mutated IBS1 and IBS2 sequences element by a nucleotide integrase comprising an Ll.*ltrB* intron RNA and the *ltrA* protein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides new methods that employ a nucleotide integrase for manipulating DNA and RNA substrates. One method uses a nucleotide integrase to cleave one strand, hereinafter referred to as the top strand, of a double-stranded DNA at a specific site and to concomitantly attach a nucleic acid molecule, which comprises an RNA molecule, to the cleaved strand at the cleavage site. The method of cleaving the top strand of a double-stranded DNA substrate comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having an EBS1 sequence and an EBS2 sequence that are capable of hybridizing with the IBS1 sequence and the IBS2 sequence, respectively on the top strand of the DNA substrate, and a group II-intron encoded protein capable of binding to or interacting with the first sequence element; and reacting the nucleotide integrase with the double-stranded DNA substrate for a time and at a temperature sufficient to permit the nucleotide integrase to cleave the top strand of the DNA substrate and to insert the group II intron RNA into the cleavage site.

The nucleotide integrase employed in this method comprises a group II intron-encoded protein bound to an excised group II intron RNA. The EBS 1 sequence and EBS2 sequence of the group II intron RNA have at least 80%, preferably 90%, more preferably full complementarity with the IBS1 sequence and IBS2 sequence, respectively, that are on the top

strand of the substrate. The group II intron-encoded protein comprises an RT domain, an X domain, and the non-conserved portion of the Zn domain.

EBS1 is located in domain I of the group II intron RNA and comprises from about 5 to 7 nucleotides that are capable of hybridizing to the nucleotides of the IBS1 sequence of the substrate. EBS2 is located in domain I of the group II intron RNA upstream of EBS1 and comprises from about 5 to 7 nucleotides that are capable of hybridizing to the nucleotides of IBS2 sequence of the substrate. If the nucleotides of the EBS1 and EBS2 sequences of the group II intron RNA are not at least 80% complementary to the nucleotides of the IBS1 or IBS2 sequences, respectively, then the group II intron RNA is modified to increase the complementarity between the EBS and IBS sequences. As shown in Fig. 1, the IBS1 sequence of the substrate is upstream of the cleavage site and the IBS2 sequence of the substrate is upstream of the IBS1 sequence.

In order to cleave the substrate efficiently, it is preferred that the nucleotide, delta, which immediately precedes the first nucleotide of EBS1 of the group II intron RNA, be complementary to the nucleotide at +1 in the top strand of the substrate. Thus, if the delta nucleotide is not complementary to the nucleotide at +1 on the top strand of the substrate, the group II intron RNA is modified to contain a delta nucleotide which is complementary to the nucleotide at +1 on the top strand of the substrate. The nucleotide integrase is then reacted with the substrate.

The DNA substrate has a recognition site which comprises a first intron RNA binding sequence that is located on the top strand of the substrate and upstream of the cleavage site and a second intron RNA binding sequence upstream that is located on the top strand of the DNA substrate and upstream of the IBS1 sequence. The recognition site also comprises a first sequence element that is located upstream of the IBS2 sequence and a second sequence element that is located downstream of the cleavage site. The first sequence element and the second sequence element both comprise from about 10 to 12 pairs of nucleotides. The first sequence element contains one or more nucleotide pairs that are required for cleavage by a particular nucleotide integrase. Except for the nucleotide pairs that are required for cleavage, it is preferred that the first sequence element comprise more A-T base pairs than G-C base pairs. It is also preferred that the second sequence element, particularly the first five pairs of nucleotides downstream from the cleavage site, be G/C poor.

Suitable nucleotide integrases for use in this method include, for example the aI2 nucleotide integrase, the aI1 nucleotide integrase, and the ItrA nucleotide integrase. The aI2 integrase is an isolated ribonucleoprotein particle that comprises a wild-type or modified group

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II intron RNA of the second intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI2 intron" RNA, bound to a wild-type or modified aI2 intron encoded-protein. The sequence of the wild-type aI2 intron RNA is set forth in SEQ.ID.NO. 1. The sequence of the protein encoded by the wild-type aI2 intron RNA is set forth in SEQ.ID.NO. 3.

5 EBS1 of the aI2 intron RNA comprises 6 nucleotides and is located at position 2985-2990 of the sequence set forth in SEQ. ID. NO. 1. EBS1 of the wild-type aI2 intron RNA has the sequence 5'-AGAAGA. EBS2 of the aI2 intron RNA comprises 6 nucleotides and is located at positions 2935-2940. EBS 2 of the wild-type aI2 intron RNA has the sequence 5'-UCAUUA.

aI2 nucleotide integrases are used to cleave double-stranded substrates that have on
 10 the top strand thereof the wt sequence, SEQ ID NO: __, shown in Fig. 3 or a target sequence that differs from the wt sequence, SEQ ID NO: ____, shown in Fig. 3. The aI2 target sequence has a T at positions -15 and -13 relative to the putative cleavage site, a C at position -18 relative to the putative cleavage site, and a G at position -16 or position -19 relative to the putative cleavage site. Thus, to use the aI2 nucleotide integrase, one first examines the sequence
 15 of the top strand of the substrate to locate a target sequence 5'GCXXTXT or a target sequence 5'XCXGTX, wherein X represents A,C,G,or T; and wherein A represents a nucleotide having an adenine base, G represents a nucleotide having a guanine base, C represents a nucleotide have a cytosine base, and T represents a nucleotide have a thymine base. Then, if the EBS2 sequence of the aI2 intron RNA does not have substantial complementarity to the
 20 IBS2 sequence, i.e., the sequence of 6 nucleotides that lie immediately downstream from one of these target sequences, and/or if EBS1 sequence of the aI2 intron RNA does not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of the IBS2 sequence, then EBS1 and EBS2 are modified to have substantial complementarity, as hereinafter explained. The efficiency of cleavage by the aI2
 25 nucleotide integrase is increased if the top strand of the substrate has an A at -21, a G at -19, a C at -18, a G at -16 a T at -15, and a T at -13.

The aI1 nucleotide integrase is an isolated ribonucleoprotein particle that comprises an excised, wild-type or modified excised group II intron RNA of the first intron of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI1 intron" RNA, and a
 30 wild-type or modified aI1 intron-encoded protein. The sequence of the aI1 intron RNA is set forth in SEQ.ID.NO. 2. The sequence of the protein encoded by the aI1 intron RNA is set forth in SEQ.ID.NO. 4. EBS1 of the aI1 intron RNA comprises 6 nucleotides and is located at position 426-431. EBS1 of the wild-type aI1 intron RNA has the sequence 5'-CGUUGA. EBS2 of the aI1 intron RNA comprises 6 nucleotides and is located at positions 376-381.

EBS2 of the wild-type aI1 intron RNA and has the sequence 5'-ACAAUU.

aI1 nucleotide integrases are used to cleave double-stranded DNA substrates that have on the top strand thereof the wt sequence, SEQ ID NO: __, shown in Fig. 6, or target sequences which differ from the wt sequence, SEQ ID NO __, shown in Fig. 6. The aI1 target
 5 sequence has C at position -13 relative to the putative cleavage site. Preferably, the top strand of the substrate has a C at -13, an A at -18, an A at -19, and a G at -21 relative to the putative cleavage site. If the EBS2 sequence of the aI1 intron RNA does not have substantial complementarity to the IBS2 sequence, i.e., the sequence of 6 nucleotides that lie immediately downstream from the C nucleotide at -13, and/or if EBS1 sequence of the aI1 intron RNA does
 10 not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of the IBS2 sequence and immediately upstream of the cleavage site, then the EBS1 sequence and the EBS2 sequence of the group II intron RNA are modified to have substantial complementarity, as hereinafter explained.

The ItrA nucleotide integrase comprises an excised, wild-type or modified excised
 15 group LI.ltrB group II intron RNA of the *Lactococcus lactis* ItrB gene, hereinafter referred to as the "LI.ltrB intron" RNA, and a wild-type or modified LI.ltrB intron-encoded protein, hereinafter referred to as the *ItrA* protein. The sequence of the LI.ltrB intron RNA is set forth in SEQ.ID.NO. 5. The sequence of the *ItrA* protein is set forth in SEQ.ID.NO. 7. The EBS1 of the LI.ltrB intron RNA comprises 7 nucleotides and is located at positions 457 to 463. The EBS1
 20 sequence of the wild-type LI.ltrB intron RNA has the sequence 5'-GUUGUGG. The EBS2 of the LI.ltrB intron RNA comprises 6 nucleotides and is located at positions 401 to and including 406. The EBS2 sequence of the wild-type LI.ltrB intron RNA has the Sequence 5'AUGUGU. The ItrA nucleotide integrase is used to cleave the top strand of a double-stranded DNA substrate when the top strand has the wt sequence, SEQ ID NO: __, shown in Fig. 8 or a target
 25 sequence which differs from the wt sequence, SEQ ID NO: __ shown in Fig. 8. The ItrA target sequence has a G at -21, an A at -20, and a T at -4 relative to the cleavage site. The ItrA nucleotide integrase cuts the top strand more efficiently when there is a G at -21, an A at -20 a T at -19, a G at -17, and a G at -15, a C at -12 and a T at -4

Another method uses a nucleotide integrase for cleaving both strands of double-
 30 stranded DNA and for attaching the group II intron RNA molecule into the cleavage site of the top strand of the DNA substrate. The nucleotide integrase comprises a group II intron-encoded protein bound to an excised group II intron RNA, wherein the group II intron RNA has an EBS1 sequence and an EBS2 sequence that have substantial complementarity to the IBS1 sequence and IBS2 sequence, respectively, on the top strand of the substrate. EBS1 comprises

from about 5 to 7 nucleotides. EBS2 comprises from about 5 to 7 nucleotides. If the nucleotides of EBS1 and EBS2 of the group II intron RNA are not at least 80% complementary to the nucleotides of IBS1 and IBS2, the non-complementary nucleotides are modified, preferably, by recombinant techniques. Preferably, the delta nucleotide of the group II intron RNA is
5 complementary to the nucleotide at +1 in the top strand. If the delta nucleotide is not complementary to the nucleotide at +1, preferably the delta nucleotide is modified to be complementary. The group II intron-encoded protein comprises an RT domain, an X domain, and the conserved and non-conserved regions of a Zn domain. To insert a cDNA into the cleavage site on the bottom strand of the substrate, the group II intron-encoded protein also
10 comprises a reverse transcriptase domain.

The method of cleaving both strands of a double-stranded DNA sequence having a recognition site comprises the steps of: providing a nucleotide integrase comprising a group II intron RNA having two sequences, EBS1 and EBS2, that are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, on the top strand of the DNA substrate, and a
15 group II-intron encoded protein that binds to a first sequence element and to a second sequence element in the recognition site of the substrate; and reacting the nucleotide integrase with the double-stranded DNA substrate for a time and at a temperature sufficient to permit the nucleotide integrase to cleave both strands of the DNA substrate and to insert the group II intron RNA into the cleavage site of the top strand. The first sequence element of the
20 recognition site is upstream of the putative cleavage site, the IBS 1 sequence and the IBS 2 sequence. The first sequence element comprises from about 10 to about 12 pairs of nucleotides. The second sequence element of the recognition site is downstream of the putative cleavage site and comprises from about from about 10 to about 12 nucleotides.

Nucleotide integrases that may be employed to cleave both strands of a DNA substrate
25 include, but are not limited to an aI2 nucleotide integrase, an aI1 nucleotide integrase, and an ItrA nucleotide integrase. The preferred recognition site for the aI2 nucleotide integrase comprises on the top strand thereof a C at -18, a T at -15, a T at -13, a G at -16 or -19, a T at +1, a T at +4, and a G at +6 relative to the cleavage site. To use the aI2 nucleotide integrase to cleave both strands of the DNA substrate, one first examines the substrate sequence to
30 determine if one strand thereof contains this set of nucleotides. Then, if the EBS2 sequence of the aI2 intron RNA does not have substantial complementarity to the IBS2 sequence of the substrate, i.e., the sequence of 6 nucleotides that lies immediately downstream from the T at -13, and/or if EBS1 sequence of the aI2 intron RNA does not have substantial complementarity to the IBS1 sequence, i.e., the sequence of six nucleotides that lie immediately downstream of

the IBS2 sequence and immediately upstream of the T at +1, then the EBS1 sequence and EBS2 sequence of the group II intron RNA are modified to have substantial complementarity, as hereinafter explained. The α 2 nucleotide integrase cleaves both strands of the substrate with greater efficiency if the top strand of the substrate has an A at -21, a G at -19, a C at -18, a G at -16, a T at -15, a T at -13 a T at +1, a T at +4, and a G at +6. The α 2 cleaves both strands of the substrate with even greater efficiency if the top strand has an A at -21, a T at -20, a G at -19, a C at -18, a T at -17, a G at -16, a T at -15, a T at -13 a T at +1, a T at +4, and a G at +6. If the top strand of the substrate additionally has a C at +2, a T at +3, a T at +7, an A at +8, an A at +9, and a T at +10, cleavage will be even greater.]

The α 1 integrase is used to cleave both strands of a DNA substrate that has on the top strand thereof a C residue at position -13 relative to the cleavage site. Preferably, the top strand of the double-stranded substrate has a C at -13, a G at -21, an A at -19, an A at -18, a T at +4, a G at +6, a T at +7, and a G at +9. Cleavage is more efficient if there is a G at -22, a G at -21, an A at -19, an A at 18, a C at -13, a T at +1, a T at +2, a T at +3, a T at +4, a A at +5, a G at +6, a T at +7, an A at +8, a G at +9, and a T at + 10 on the top strand of the DNA substrate. If the top strand of the substrate additionally comprises a T at -20, a T at -17, a T at -16, a C at -15, and an A at -14, cleavage will be even greater.

The ItrA nucleotide integrase is used to cleave both strands of a double stranded DNA substrate, when the substrate has on the top strand thereof a G at -21, an A at -20 a C +1, an A at +2, a T at +3, an A at +4, a T at +5, a C at +6, an A at +7, and a T at +8. The ItrA nucleotide integrase cleaves both strands of the substrate more efficiently if the top strand has a G at -21, an A at -20, a T at -19, a G at -17, and G at -15, a C +1, an A at +2, a T at +3, an A at +4, a T at +5, a C at +6, an A at +7, and a T at +8. If the top strand additionally has a C at -22, a C at -18, a T at -16, an A at -14, an A at -13, a T at +9 and a T at +10, cleavage will be even greater.

Another method uses a nucleotide integrase for cleaving a single-stranded nucleic acid substrate, i.e., a single-stranded DNA or RNA, and for attaching the group II intron RNA molecule into the cleavage site. The method comprises the steps: providing a nucleotide integrase comprising: a group II intron RNA having two hybridizing sequences, EBS1 and EBS2, which are capable of hybridizing with two intron RNA-binding sequences, IBS1 and IBS2, respectively on the substrate, and a group II intron encoded protein having an RT domain, an X domain and the non-conserved portions of the Zn domain; and reacting the substrate with the nucleotide integrase. The EBS1 sequence of the group II intron RNA comprises from about 5 to 7 nucleotides and has at least 80%, preferably 90%, and more preferably full complementarity with the nucleotides at positions -1 to about -5 or about -7.

The EBS2 sequence of the group II intron RNA comprises 4 to 7 nucleotides and has at least 80%, preferably 90%, more preferably full complementarity with the nucleotides at positions from about -6 to about -13. Preferably, the nucleotide immediately preceding the first nucleotide of EBS1 is complementary to the nucleotide at +1 in the sense strand. More preferably, the three nucleotides immediately upstream of EBS 1 are complementary to nucleotides +1 to +3 on the single-stranded substrate.

The present invention also provides a method of determining whether a nucleic acid substrate comprises a particular recognition site. The method comprises the steps of providing a nucleotide integrase capable of cleaving a nucleic acid substrate with a particular recognition site; reacting the nucleotide integrase with the nucleic acid substrate; and assaying for cleavage of the substrate. Cleavage of the substrate indicates that the substrate comprises the particular recognition site. In addition to assaying for fragmentation and alterations in size of the nucleic acid substrate, cleavage may be detected by assaying for incorporation into or attachment of the group II intron RNA to one strand of the nucleic acid substrate.

While a wide range of temperatures are suitable for the methods herein, good results are obtained at a reaction temperature of from about 30°C to about 42°C, preferably from about 30° to about 37°C. A suitable reaction medium contains a monovalent cation such as Na⁺ or K⁺, at a concentration from about 0 to about 300 mM; preferably from about 10 to about 200 mM KCl, and a divalent cation, preferably a magnesium or manganese ion, more preferably a magnesium ion, at a concentration that is less than 100 mM and greater than 1 mM. Preferably the divalent cation is at a concentration of about 5 to about 20mM, more preferably about 10 to about 20 mM. The preferred pH for the medium is from about 6.0-8.5, more preferably about 7.5-8.0

In the above-described methods it is believed that the single stranded nucleic acid substrates and the top strand of the double-stranded DNA substrate are cut by the excised group II intron RNA. The cleavage that is catalyzed by the excised group II intron RNA is a reverse splicing reaction that results in the insertion, either partially or completely, of the excised group II intron RNA into the cleavage site, i.e. between nucleotides -1 and +1 in the top strand. With partial insertion the group II intron RNA is covalently attached to the +1 nucleotide on the top strand of the cleavage site. It is believed that the bottom strand or antisense strand of the double-stranded DNA substrate is cut by the group II intron-encoded protein. The bottom strand of the double stranded DNA substrate is cut at a position from about 9 to about 11 base pairs downstream of the cleavage site in the top strand, i.e., at a site between nucleotides positions +9, +10, and +11.

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The methods of using a nucleotide integrase as an endonuclease to cleave a substrate DNA are useful analytical tools for determining the presence and location of a particular recognition site in a DNA substrate. Moreover, the simultaneous insertion of a nucleic acid molecule into the DNA substrate, which occurs when either single-stranded DNA or double stranded DNA is cleaved with a nucleotide integrase, permits tagging of the cleavage site of the DNA substrate with a radiolabeled molecule, a feature which facilitates in identifying DNA substrates that contain a particular recognition site. In addition, the automatic attachment of an RNA molecule onto one strand of a double-stranded DNA substrate permits identification of the cleavage site through hybridization studies that use a probe that is complementary to the attached RNA molecule. An attached RNA molecule that is tagged with a molecule such as biotin also enables the cleaved strand to be affinity purified.

The methods of using nucleotide integrases to cleave RNA and DNA substrates having a recognition site are useful for rendering certain genes within the substrates nonfunctional. Such methods are also useful for inserting a nucleic acid into the cleavage site, thus, changing the characteristics of the RNA molecules and the protein molecules encoded by the substrates.

The nucleotide integrase

The nucleotide integrase is an isolated ribonucleoprotein ("RNP") particle and comprises a group II intron encoded RNA and a group II intron encoded protein, which protein is bound to the RNA. Preferably, the group II intron RNA is an excised group II intron RNA. "Excised group II intron RNA," as used herein, refers to an RNA that is either an in vitro or in vivo transcript of the DNA of the group II intron and that lacks flanking exon sequences. The excised group II intron RNA is obtained from wild type organisms, or mutated organisms, by in vivo transcription and splicing, or by in vitro transcription and splicing from the transcript of a modified or unmodified group II intron. "Group II intron encoded protein" as used herein, is a protein encoded by a group II intron open reading frame.

A group II intron is a specific type of intron that is present in the DNA of bacteria and in the DNA of organelles, particularly the mitochondria of fungi, yeast and plants and the chloroplast of plants. The group II intron RNA molecules, that is, the RNA molecules which are encoded by the group II introns, share a similar secondary and tertiary structure. Figure 2 depicts the secondary structure of the aI1 and aI2 intron RNA and part of the nucleotide sequence of the wild-type aI1 and aI2 intron RNA. The group II intron RNA

molecules typically have six domains. Domain IV of the group II intron RNA contains the nucleotide sequence which encodes the "group II intron encoded protein."

Nucleotide integrases include, for example, excised group II intron RNA molecules having a sequence which is identical to a group II intron RNA that is found in nature, i.e. a wild-type group II intron RNA, and excised group II RNA's which have a sequence different from a group II intron RNA that is found in nature, i.e. a modified, excised group II intron RNA molecule. Modified excised group II intron RNA molecules, include, for example, group II intron RNA molecules that have nucleotide base changes or additional nucleotides in the internal loop regions of the group II intron RNA, preferably the internal loop region of domain IV and group II intron RNA molecules that have nucleotide base changes in the hybridizing regions of domain I. Nucleotide integrases in which the group II intron RNA has nucleotide base changes in the hybridizing region, as compared to the wild type, typically have altered specificity for the substrate DNA of the nucleotide integrase.

The group II intron-encoded protein of the nucleotide integrase comprises an X domain and a Zn domain. The X domain of the protein has a maturase activity. The Zn domain of the protein has Zn^{2+} finger-like motifs. Preferably, the group II intron-encoded protein further comprises a reverse transcriptase domain. As used herein, a group II intron-encoded protein includes modified group II intron-encoded proteins that have additional amino acids at the N terminus, or C terminus, or alterations in the internal regions of the protein as well as wild-type group II intron-encoded proteins. It is believed that the group II intron-encoded protein is bound to 3' region of the group II intron RNA.

The nucleotide integrase are provided in the form of RNP particles isolated from wild-type, mutant, or genetically-engineered organisms. The nucleotide integrase are also provided in the form of reconstituted RNP particles isolated from a reconstituted RNP particle preparation. The nucleotide integrase also comprises reconstituted RNP particles that are formed by combining an exogenous synthetic, excised group II intron RNA with either a group II intron-encoded protein or an RNA-protein complex preparation. The exogenous RNA includes both unmodified and modified group II intron RNA molecules. Preferably, the exogenous RNA is an in vitro transcript or a derivative of an in vitro transcript of an unmodified or modified intron group II intron. For example, the exogenous RNA may be derived by splicing from an in vitro transcript. The RNA-protein complex preparation contains group II intron-encoded protein molecules complexed to RNA molecules that are not an excised group II RNA molecule having a sequence which encodes this protein. The

group II intron-encoded protein of the RNA-protein complex is associated with either a ribosomal RNA molecule, an mRNA molecule, or an excised group II intron RNA that does not encode the group II-intron encoded protein.

The nucleotide integrase may be used as a purified RNP particle or a purified reconstituted particle. Alternatively, the nucleotide integrase may be used in a partially-purified preparation which contains the RNP particles and reconstituted particles that have nucleotide integrase activity as well as other RNP particles, such as for example ribosomes. This partially-purified preparation is free of organelles.

Preparation of the Nucleotide Integrase

The nucleotide integrase is isolated from wild type or mutant yeast mitochondria, fungal mitochondria, plant mitochondria, chloroplasts, the proteobacterium *Azotobacter vinelandii*, the cyanobacterium *Calothrix*, and *Escherichia coli lactococcus lactis*. The procedure for isolating the RNP particle preparation involves mechanically and/or enzymatically disrupting the cell membranes and/or cell walls of the organisms. In the case of fungi and plants, the purification also involves separating the specific organelles, such as mitochondria or chloroplasts, from the other cellular components by differential centrifugation and/or flotation gradients and then lysing the organelles with a nonionic detergent, such as Nonidet P-40. The organelle and bacterium lysates are then centrifuged through a sucrose cushion to obtain the ribonucleoprotein (RNP) particle preparation. The RNP particles may be further purified by separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography.

The nucleotide integrase is also isolated from reconstituted RNP particle preparations that are prepared by combining an RNA-protein complex preparation with an exogenous, excised group II intron RNA. The RNA-protein complex preparation is preferably isolated from a yeast, fungi, or bacterium using the protocol for RNP particles described above. The RNA-protein complex preparation comprises group II intron-encoded protein molecules complexed with RNA molecules that are not an excised group II intron RNA having a sequence that encodes the group II intron-encoded protein. The group II intron-encoded protein of the RNA-protein complex preparation is associated with either a ribosomal RNA molecule, an mRNA molecule, or an excised group II intron RNA that does not encode the group II-intron encoded protein.

The exogenous RNA preferably is a synthetic molecule made by in vitro transcription or by in vitro transcription and self-splicing of the group II intron. The exogenous RNA may also be made by isolation of the group II intron RNA from cells or

organelles in which it is naturally present or from cells in which an altered intron has been inserted and expressed. The exogenous RNA is then added to a preparation containing the RNA-protein complex. Preferably, the exogenous group II intron RNA is first denatured. The exogenous RNA is added to the RNA-protein complex on ice.

5 In another embodiment, the nucleotide integrase is made by introducing an isolated DNA molecule which comprises a group II intron DNA sequence into a host cell. Suitable DNA molecules include, for example, viral vectors, plasmids, and linear DNA molecules. Following introduction of the DNA molecule into the host cell, the group II intron DNA sequence is expressed in the host cell such that excised RNA molecules encoded
10 by the introduced group II intron DNA sequence and protein molecules encoded by introduced group II intron DNA sequence are formed in the cell. The excised group II intron RNA and group II intron-encoded protein are combined within the host cell to produce the nucleotide integrase.

Preferably the introduced DNA molecule also comprises a promoter, more
15 preferably an inducible promoter, operably linked to the group II intron DNA sequence. Preferably, the DNA molecule further comprises a sequence which encodes a tag to facilitate isolation of the nucleotide integrase such as, for example, an affinity tag and/or an epitope tag. Preferably, the tag sequences are at the 5' or 3' end of the open reading frame sequence. Suitable tag sequences include, for example, sequences which encode a series of histidine
20 residues, the Herpes simplex glycoprotein D, i.e., the HSV antigen, or glutathione S-transferase. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker. Optionally, the DNA molecule comprises sequences that encode molecules that modulate expression, such as for example T7 lysozyme.

The DNA molecule comprising the group II intron sequence is introduced into
25 the host cell by conventional methods, such as, by cloning the DNA molecule into a vector and by introducing the vector into the host cell by conventional methods, such as electroporation or by CaCl_2 -mediated transformation procedures. The method used to introduce the DNA molecule is related to the particular host cell used. Suitable host cells are those which are capable of expressing the group II intron DNA sequence. Suitable host cells
30 include, for example, heterologous or homologous bacterial cells, yeast cells, mammalian cells, and plant cells. In those instances where the host cell genome and the group II intron DNA sequence use different genetic codes, it is preferred that the group II intron DNA sequence be modified to comprise codons that correspond to the genetic code of the host cell. The group II intron DNA sequence, typically, is constructed de novo from synthetic

oligonucleotides or modified by *in vitro* site-directed mutagenesis to prepare a group II intron DNA sequence with different codons. Alternatively, to resolve the differences in the genetic code of the intron and the host cell, DNA sequences that encode the tRNA molecules which correspond to the genetic code of the group II intron are introduced into the host cell.

- 5 Optionally, DNA molecules which comprise sequences that encode factors that assist in RNA or protein folding, or that inhibit RNA or protein degradation are also introduced into the cell.

The DNA sequences of the introduced DNA molecules are then expressed in the host cell to provide a transformed host cell. As used herein the term "transformed cell" means a host cell that has been genetically engineered to contain additional DNA, and is not limited to cells which are cancerous. Then the RNP particles having nucleotide integrase activity are isolated from the transformed host cells.

Preferably, the nucleotide integrase is isolated by lysing the transformed cells, such as by mechanically and/or enzymatically disrupting the cell membranes of the transformed cell. Then the cell lysate is fractionated into an insoluble fraction and soluble fraction. Preferably, an RNP particle preparation is isolated from the soluble fraction. RNP particle preparations include the RNP particles having nucleotide integrase activity as well as ribosomes, mRNA and tRNA molecules and other RNPs. Suitable methods for isolating RNP particle preparations include, for example, centrifugation of the soluble fraction through a sucrose cushion. The RNP particles, preferably, are further purified from the RNP particle preparation or from the soluble fraction by, for example, separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography. For example, in those instances where the protein component of the desired RNP particle has been engineered to include a tag such as a series of histidine residues, the RNP particle may be further purified from the RNP particle preparation by affinity chromatography on a matrix which recognizes and binds to the tag. For example, NiNTA Superflow from Qiagen, Chatsworth CA, is suitable for isolating RNP particles in which the group II intron-encoded protein has a His₆ tag.

The following methods for preparing nucleotide integrases are included for purposes of illustration and are not intended to limit the scope of the invention.

FORMULATIONS

30 The RNP particle preparations of the following formulations 1-10, and the RNA-protein complex of the formulation 12 were isolated from the mitochondria of the wild-type *Saccharomyces cerevisiae* yeast strain ID41-6/161 *MATa adel lys1*, hereinafter designated "161", and derivatives thereof. The mitochondria of the wild-type yeast strain 161 contains a *COX1* gene that includes the group II intron a11 and the group II intron a12.

The *COX1* gene in the mutant yeast strains either lacks one of the group II introns or has a mutation in one of the group II introns. The excised group II intron RNA molecules and the group II intron encoded proteins are derived from the group II introns a11 and a12 that are present in the wild-type and mutant yeast strains.

5 The intron composition of the *COX1* gene in the different yeast strains is denoted by a convention in which a superscript "+" indicates the presence of the a11 intron or the a12 intron, a superscript "0" indicates the absence of the a11 or a12 intron, and other superscripts refer to specific alleles or mutations in the a12 intron.

Formulation 1

10 An RNP particle preparation was isolated from the mitochondria of the *Saccharomyces cerevisiae* wild-type yeast strain 161. The intron composition of the *COX1* gene of the wild-type strain is 1⁺2⁺. The RNP particle preparation contains an RNP particle that is derived from the a11 intron and includes an excised a11 RNA bound to a protein encoded by a11. The preparation also contains an RNP particle that is derived from the a12
15 intron and that comprises a excised a12 RNA molecule and an associated a12-encoded protein.

To prepare the RNP particle preparation, the yeast were inoculated into a 1 liter liquid culture medium containing 2% raffinose, 2% BactoPeptone from Difco and 1% yeast extract from Difco to an O.D.₅₉₅ of 1.6-1.7. The cell walls were digested with 40 mg of
20 the yeast lytic enzyme from ICN, and the cells broken by mechanical disruption with glass beads. The nuclei and cell debris were pelleted from the lysate by centrifugation for 5 minutes in a Beckman GSA rotor at 5,000 rpm. The supernatant was removed and centrifuged in a Beckman GSA rotor at 13,000 rpm for 15 minutes to obtain a mitochondrial pellet. The mitochondria were layered on a flotation gradient consisting of a 44% sucrose
25 solution layer, a 53% sucrose solution layer, and a 65% sucrose solution layer and centrifuged in a Beckman SW28 rotor at 27,000 rpm for 2 hours and 10 minutes. The mitochondria were collected from the 53%/44% interface and suspended in buffer containing 0.5M KCl, 50 mM CaCl₂, 25 mM Tris-HCl, pH 7.5, 5mM DTT and lysed by the addition of Nonidet P-40 to a final concentration of 1%. The mitochondrial lysate was then centrifuged
30 in a Beckman 50Ti rotor at 50,000 rpm for 17 hours through a 1.85 M sucrose cushion in a buffer containing 0.5M KCl, 25 mM CaCl₂, 25 mM Tris-HCl, pH 7.5, 5mM DTT, to obtain a pellet of RNP particles that were largely free of mitochondrial proteins. The isolated RNP particles were resuspended in 10 mM Tris-HCl, pH 8.0 and 1 mM DTT and stored at -70°C. The preparation may be repeatedly thawed and frozen before use.

Formulation 1a Purified RNP particle

2.5 O.D.₂₆₀ of the RNP particles from formulation 1 in a volume of 150 μ l were layered onto a 12 ml 5-20% linear sucrose gradient in a buffer consisting of 100 mM KCl, 2 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and 5 mM DTT. The gradient was centrifuged
5 in an SW41 rotor at 4°C at 40,000 rpm for five hours. The gradient was fractionated into 35 fractions of approximately 0.325 ml. Fractions 12-20 contain the purified RNP particles which are substantially free of ribosomal RNA. The location of the RNP particles in the gradient fractions was independently verified by Northern hybridization with aI2 antisense RNA. The location of the small and large subunits of ribosomal RNA in the gradient
10 fractions was independently verified by ethidium bromide staining of the fractions on a 1% agarose gel. Approximately 85% of the ribosomal RNA is found in a fraction that does not contain the RNP particles which comprise the nucleotide integrase.

Formulation 2 RNP particle preparation from mutant yeast strain 1⁰²⁺

The RNP particles comprise an excised aI2 RNA and an aI2-encoded protein.
15 Yeast strain 1⁰²⁺ was obtained from Dr. Philip S. Perlman at the University of Texas Southwestern Medical Center and was prepared as described in Moran et al., 1995, Mobile Group II Introns of Yeast Mitochondrial DNA Are Novel Site-Specific Retroelements, Mol. Cell Biol. 15, 2828-38, which is incorporated herein by reference. The 1⁰²⁺ mutant strain was constructed as follows: (i) the aI2 intron from strain 161 was cloned as a *Clal*-to-*Bam*HI fragment into pBluescript KS⁺ obtained from Stratagene to yield pJVM4; (ii) pJVM4 was
20 cleaved with *Clal* and *NdeI* to remove the 5' end of the insert; and (iii) an *MspI*-to-*NdeI* fragment that contains exons 1 and 2 of the mitochondriae *COX1* gene plus the 5' end of aI2 from yeast strain C1036 Δ I was inserted to yield plasmid pJVM164. Yeast strain C1036 Δ I, in which aI1 is excised from the mitochondrial DNA, was prepared as described in Kennell et
25 al., 1993, Reverse transcriptase activity associated with maturase-encoding group II introns in yeast mitochondria. Cell 73, 133-146, which is incorporated herein by reference. pJVM164 was transformed into a [*rho*⁰] strain, and the 1⁰²⁺ allele was placed into an intact mitochondrial DNA by recombination. This last step is accomplished by mating to a nonreverting *COX1* mutant derived from mutant C1036 (strain 5B), whose construction is
30 described in Kennell et al., 1993, and selecting for recombinant progeny that are capable of respiring and growing on glycerol-containing medium (GLY⁺) and that contain the transformed *COX1* allele in place of the 5B allele.

The reactions and manipulations directed at cloning DNA, such as ligations, restriction enzyme digestions, bacterial transformation, DNA sequencing etc. were carried out according to standard techniques, such as those described by Sambrook et al., Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press. Cold Spring Harbor. N.Y. Yeast mitochondrial transformations were also carried out according to standard techniques such as those described in Belcher et al., 1994, Biolistic transformation of mitochondria in *Saccharomyces cerevisiae*, 101-115. In N.-S. Yang and P. Christou (ed.) Particle Bombardment Technology for Gene Transfer. Oxford University Press, New York. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1[°]2⁺, as in formulation 1.

Formulation 3 RNP particle preparation from mutant yeast strain 1[°]2[°]

Yeast strain 1[°]2[°] is a derivative of the wild-type yeast strain 161. The yeast strain 1[°]2[°] was obtained from Dr. Philip S. Perlman and was prepared as described in Kennell et al., 1993. Cell 73, 133-146. Yeast strain 1[°]2[°] contains a segment of the COX1 gene of *S. diastaticus*, which lacks aI2, inserted into wild-type 161 mtDNA via mitochondrial transformation. The construction started with plasmid pSH2, which contains aI1 from wild-type 161 and some flanking sequences cloned as a *HpaII/EcoRI* fragment in pBS(+) (Stratagene, La Jolla, CA). That plasmid was cleaved near the 3' end of aI1 with *ClaI* and in the downstream polylinker with *BamHI*, and the gap was filled with a *ClaI/BamHI* fragment from *S. diastaticus* mitochondrial DNA (NRRL Y-2416) that contains the 3' end of aI1, E2, E3 and most of aI3, thus creating a 1[°]2[°] form of the COX1 gene. The plasmid containing the hybrid COX1-1[°]2[°] segment was transformed into a rho[°] derivative of strain MCC109 (MATa ade2-101 ura3-52 kar1-1) by biolistic transformation. The resulting artificial petite was crossed to strain n161/m161-5B, and gly⁺ recombinants containing the COX1 1[°]2[°] allele in the n161 background were isolated. The hybrid aI1 allele, which is spliced normally, differs from that of wild-type 161 by one nucleotide change, C to T, at position 2401, changing Thr₇₄₄ to Leu in the intron open reading frame. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1[°]2[°] as in formulation 1. The RNP particles comprise an excised aI1 RNA molecule and an aI1 encoded protein.

Formulation 4 RNP particle preparation from mutant yeast strain 1[°]2^{YAHH}

Yeast strain 1[°]2^{YAHH} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995, Mol. Cell Biol. 15, 2838-38., using a mutagenized pJVM164 plasmid. The allele was made by oligonucleotide-directed mutagenesis of pJVM164 which contains a 4.4 kb *MspI/BamHI* fragment extending from 217 nucleotides

upstream of exon 1 through intron aI3 of the COX1 allele. The mutagenesis changes the aI2 nucleotides 1473 to 1478 from GAT GAT to CAT CAT (D-491D-492 to HH). The RNP particles comprise a mutated, excised aI2 RNA and an aI2-encoded protein that has the mutation YADD@YAAH in the reverse transcriptase domain of the protein. The RNP particle preparation was made from the mitochondria of mutant yeast strain 1°2^{YAAH} as in formulation 1.

Formulation 5 RNP particles from the mutant yeast strain 1°2^{P714T}

The mutant yeast strain 1°2^{P714T} was obtained from Dr. Philip S. Perlman and was constructed according to the procedure described in Kennell et al., 1993, Cell 73, 133-146, where it is named n161/m161-C1036Δ1. The RNP particles comprise a mutated, excised aI2 intron RNA molecule and an aI2-encoded protein that carries the missense mutation P₇₁₄T in the Zn domain. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{P714T} as in formulation 1.

Formulation 6 RNP particle from mutant yeast strain 1°2^{HHVR}

The mutant yeast strain 1°2^{HHVR} was obtained from Dr. Philip S. Perlman and was made by using the nucleotide described in Moran et al., 1995, Mol. Cell Biol. 15, 2828-38, which is incorporated herein by reference, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The aI2 intron has the following changes: positions 2208-2219 from CATCACGTAAGA SEQ. ID. NO. 9 to GCAGCTGCAGCT, (H₇₃₆H₇₃₇V₇₃₈R₇₃₉ to AAAA) and A₂₂₂₇ A to T (N₇₄₂I). This nucleotide integrase preparation comprises a mutated, excised aI2 intron RNA and an aI2-encoded protein that has a missense mutation in the HHVR motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{HHVR}.

Formulation 7 RNP particle from mutant yeast strain 1°2^{ΔConZn}

The mutant yeast strain 1°2^{ΔConZn} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995, Mol. Cell Biol. 15, 2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by oligonucleotide-directed mutagenesis of pJVM164. The aI2 intron has the following changes: positions 2157-2165 changed from TTATTTAGT to TAATAATAA (L₇₁₉F₇₂₀S₇₂₁ to OchOchOch). RNP particles comprise a mutated, excised aI2 intron RNA and an aI2-encoded protein that lacks the most conserved motifs in the Zn domain. The RNP particle preparation was made from mitochondria of mutant yeast strain 1°2^{ΔConZn}.

Formulation 8 RNP particle from mutant yeast strain 1^{02C-C/1}

The mutant yeast strain 1^{02C-C/1} was obtained from Dr. Phillip S. Perlman and was made by using a nucleotide described in Moran et al., 1995 Mol. Cell Biol. 15, 2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The aI2 intron has the following changes: positions 2172-2173 changed from TG to GC (C₇₂₄A) and 2180-2182 changed from TTG to AGC (I₇₂₆C₇₂₇ to MA). The RNP particles comprise a mutated, excised aI2 intron RNA and an aI2-encoded protein that has three amino acid residues changed in the first Zn⁺²-finger-like motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1^{02C-C/1}.

Formulation 9 RNP particles from mutant yeast strain 1^{02C-C/2}

The mutant yeast strain 1^{02C-C/2} was obtained from Dr. Philip S. Perlman and was made as described in Moran et al., 1995 Mol. Cell Biol. 15, 2828-38, using a mutagenized pJVM164 plasmid. The allele was constructed by site-directed mutagenesis of pJVM164. The aI2 intron has the following changes: position 2304-2305 changed from TG to GC (C₇₆₈A) and 2313-2314 changed from TG to GC (C₇₇₁A). The RNP particles comprise a mutated excised aI2 intron RNA and an aI2-encoded protein that has two amino acids changed in the second Zn⁺² finger-like motif. The RNP particle preparation was made from mitochondria of mutant yeast strain 1^{02C-C/2}.

Formulation 10 RNP particles from mutant yeast strain 1^{02H6}

The mutant yeast strain, obtained from Dr. Philip S. Perlman, was made by transferring the mutagenized plasmid pJVM164 into the mitochondria of yeast strain GRF18 as described in Moran et al., 1995 Ref. The allele was constructed by site-directed mutagenesis of pJVM164 and has the sequence CATCATCATCATCAT, SEQ. ID. NO. 10, inserted between nucleotides 2357 and 2358 of the aI2 intron. The RNP particle preparation was made from mitochondria of mutant yeast strain 1^{02H6} according to the protocol described above for formulation 1. The RNP particles comprise a mutated, excised aI2 intron RNA and an aI2-encoded protein that has six histidines added to the C terminus of the aI2-encoded protein.

Formulation 11 RNP particles from Neurospora intermedia.

Mitochondria from the Varkud strain of Neurospora intermedia, which is available from the Fungal Genetics Stock Center, were prepared as described in Lambowitz A.M. 1979, Preparation and analysis of mitochondrial ribosomes. Meth. Enzymol. 59, 421-433. The conidia were disrupted with glass beads and the mitochondria and RNP particles

isolated as described in formulation 1. The RNP particles comprise an excised *col* intron RNA and the protein encoded by the *col* intron.

Formulation 12 Reconstituted RNP particle preparation

A reconstituted RNP particle preparation was made by incubating an
5 exogenous, excised, in vitro RNA transcript of the aI2 intron with an RNA-protein complex preparation isolated from the mutant yeast strain $1^{02\Delta D5}$, in which the aI2 intron RNA lacks a domain V and is therefore splicing defective. The mutant allele $1^{02\Delta D5}$ was obtained from Dr. Philip S. Perlman and was constructed using the same procedure that was used to make yeast strain $1^{+2\Delta D5}$ that was described in Moran et al. 1995, except that the final mating was with
10 yeast strain 1^{02+} . The RNA-protein complex preparation was isolated from $1^{02\Delta D5}$ using the protocol described above in formulation 1 for RNP particle preparations. The RNA-protein complex preparation isolated from the mitochondria of $1^{02\Delta D5}$ does not contain excised aI2 RNA but does contain aI2-encoded protein that is associated with other RNA molecules in the preparation.

15 The exogenous RNA was made by in vitro transcription of the plasmid pJVM4 which includes a fragment of the yeast mitochondrial *COX1* gene from the *Clal* site of the group II intron 1 (aI1) to the *BamHI* site of aI3 that has been inserted into the pBLUESCRIPT KS+ plasmid. Plasmid pJVM4 contains the following *COX1* sequences: Exon 2, aI2, Exon 3 and parts of aI1 and aI3 sequence. The sequences are operably linked to
20 a T3 RNA polymerase promoter. The Exon 2 and Exon 3 sequence are required for self-splicing of the aI2 intron RNA from the RNA transcript. pJVM4 was linearized with *BstEII*, which cuts at the 3' end of Exon 3 then 5 µg of the plasmid was incubated in 0.300 ml of 40 mM Tris-HCl at pH 8.0, 25 mM NaCl, 8 mM MgCl₂ 2 mM spermidine, 5 mM DTT 500 mM rNTPs, 600 U of RNasin from US Biochemical and 300-750 U of T3 RNA polymerase from
25 BRL at 37°C for 2 hours to make the RNA transcripts. Following the incubation, the RNA transcripts were phenolcia extracted, purified on G-50 column, phenolcia extracted and precipitated with ethanol. The RNA transcripts were then incubated in 40 mM Tris-HCl at pH 7.5, 100 mM MgCl₂, 2 M NH₄Cl at 40-45°C for 1 hour to allow self-splicing of the aI2 intron RNA molecules from the RNA transcripts and to obtain the splicing products. The
30 splicing products, which include the excised aI2 RNA transcript, the ligated transcript which lacks the aI2 intron RNA, and the unspliced transcript, were desalted by passing through a G-50 column, then phenolcia extracted and ethanol precipitated to provide the exogenous

RNA. The exogenous RNA was then resuspended to a final concentration of 1.0 mg/ml in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

To prepare the reconstituted RNP particle preparation, 1 μ l of the exogenous RNA was added to 2 μ l of the 1^o2^{AD5} RNA-protein complex preparation (0.025 O.D.₂₆₀ units) on ice for 0-10 minutes. The preparation was used immediately.

Formulation 13. Reconstituted RNP Particle Preparation containing a Nucleotide Integrase Comprising a Group II Intron RNA Having Modified EBS Sequences

Plasmid pJVM4 derivatives were used to prepare exogenous aI2 intron RNA molecules in which the EBS1 and EBS2 sequences are different from the EBS sequences in the wild-type aI2 intron. pJVM4 contains the aI2 intron sequence and flanking exon sequences from wild-type yeast 161 cloned downstream of a phage T3 promoter in pBluescript II KS(+). Plasmids containing modified introns were derived from pJVM4 by PCR mutagenesis with appropriate primers. In all cases, the modified region was sequenced to verify the correct mutation and the absence of adventitious mutations.

Plasmids pJVM4-aI1EBS1, pJVM4-aI1EBS2 and pJVM4-aI1EBS1/EBS2 contain aI2 RNA derivatives in which the EBS1 and/or EBS2 sequences were replaced with those of aI1. In each case, portions of the 5' and 3' exons were also changed to aI1 sequences to permit in vitro splicing. pJVM4-aI1EBS1 has EBS1 positions 2985-2990 changed from 5'AGAAGA to 5'CGTTGA; pJVM4-aI1EBS2 has EBS2 positions 2935-2940 changed from 5' TCATTA to 5' ACAATT; and pJVM4-aI1EBS1EBS2 has EBS1 and EBS2 positions 2935-2940 and 2985-2990 changed from 5' TCATTA to 5' ACAATT and 5' AGAAGA to 5' CGTTGA, respectively. For pJVM4-aI1EBS1 and pJVM4-aI1EBS1/EBS2, the 5' portion of the pJVM4 insert consisting of aI1 and E2 sequence was replaced with the last 24 bp of E1. For pJVM4-aI1EBS2, positions -24 to -7 (GTCATGCTGTATTAATGA) SEQ. ID. NO. 11 were replaced with (ATGGTAATTCACAATTAT), SEQ. ID. NO. 12 leaving the aI2 IBS1 sequence unchanged. For all three constructs, the 3' portion of the insert was replaced by the first 15 bp of E2 instead of E3 and aI3.

pJVM4-EBS2-8G, , pJVM4-EBS2-9T-10A, pJVM4-EBS2-11A, pJVM4-EBS2-12T, and pJVM4-EBS2-13T(1) are derivatives of pJVM4 in which the indicated changes were introduced at different positions in EBS2. pJVM4-EBS2-13T(2) is identical to pJVM4-EBS2-13T(1) except that it contains a second mutation, T to A, at intron position 2932.

pJVM4- δ -C, pJVM4- δ -G, and pJVM9- δ -T are derivatives of pJVM4 in which the δ nucleotide (position 2984) was changed to C, G, or T, respectively, with the compensatory nucleotide substituted at the δ' position of exon 3 for in vitro splicing.

Exogenous aI2 intron transcripts having a modified EBS1 sequence and/or a modified EBS2 sequence were synthesized using phage T3 polymerase and the modified plasmids as templates. The synthetic transcripts contained regions of the modified aI2 intron RNA and regions of the flanking exon 2 and exon 3 of the yeast mitochondrial COX1 protein. The synthetic transcripts were self-spliced and the spliced products desalted through a G-50 column, phenol-CIA extracted, ethanol precipitated, and dissolved in TE (pH8.0) at a final concentration of 1.0 $\mu\text{g}/\mu\text{l}$ (0.52 μM).

The resulting modified, excised aI2 RNA molecules were individually mixed with RNA-protein complex preparations isolated from 1⁰2^{AD5} using the protocol described above in formulation 1 for RNP particle preparations. This yeast mutant has a deletion in domain V of the aI2 intron and is unable to splice aI2 RNA. This mutant overproduces aI2 protein from the unspliced precursor mRNA. Thus, the RNA-protein complex preparation contains larger amounts of the aI2 protein.

For reconstitution, 1 μl of the spliced, synthetic aI2 transcripts was mixed with 2 μl (0.025OD₂₆₀ units) of the RNA-protein complex preparation and incubated on ice for 0-10 minutes.

20 Formulation 14

An RNP particle preparation containing an RNP particle in which the loop region of domain IV of the group II intron RNA is modified, that is the loop region nucleotide sequence of domain IV differs from the nucleotide sequence of the aI2 RNA of formulations 1-10 is prepared by two methods. First oligonucleotide-directed mutagenesis of the aI2 intron DNA is performed by standard, well-known methods to change the nucleotide sequences which encode for the loop region of domain IV of the aI2 intron RNA. The mutagenized aI2 intron DNA is then inserted into a vector, such as a plasmid, where it is operably linked to an RNA polymerase promoter, such as a promoter for T7 RNA polymerase or SP6 RNA polymerase or T3 RNA polymerase and an in vitro transcript of the modified group II intron RNA is made as described above in formulation 12. The exogenous RNA is then combined with an RNA-protein complex that has been isolated as described for formulation 12 to produce a modified reconstituted RNP particle preparation.

Alternatively, an RNP particle preparation in which the sequences within the loop region of the group II intron RNA are modified is prepared by site-directed mutagenesis of an organism, such as a yeast, as described in formulations 4-10, and by isolation of the RNP particle preparation from the organism as described in formulation 1. IV is performed as in formulations 4-10 of the *al2* intron DNA.

Formulation 15 RNP Particle Preparation from a Genetically-Engineered Cell

A nucleotide integrase comprising an excised RNA which is encoded by the Ll.ltrB intron of a lactococcal conjugative element pRS01 of *Lactococcus lactis* and the protein encoded by the ORFLtrA of the Ll.ltrB intron were prepared by transforming cells of the BLR(DE3) strain of the bacterium *Escherichia coli*, which has the *recA* genotype, with the plasmid pETLtrA19. Plasmid pETLtrA19 comprises the DNA sequence for the group II intron Ll.ltrB from *Lactococcus lactis*, positioned between portions of the flanking exons *ltrBE1* and *ltrBE2*. pETLtrA19 also comprises the DNA sequence for the T7 RNA polymerase promoter and the T7 transcription terminator. The sequences are oriented in the plasmid in such a manner that the ORF sequence, SEQ. ID. NO. 6, within the Ll.ltrB intron is under the control of the T7 RNA polymerase promoter. The ORF of the Ll.ltrB intron encodes the protein *ltrA*. The sequence of the Ll.ltrB intron and the flanking exon sequences present in pETLtrA19 are shown SEQ.ID. NO. 5. The amino acid sequence of the *ltrA* protein is shown in SEQ. ID. NO.7. Domain IV is encoded by nucleotide 705 to 2572.

pETLtrA19 was prepared first by digesting pLE12, which was obtained from Dr. Gary Dunny from the University of Minnesota, with *HindIII* and isolating the restriction fragments on a 1% agarose gel. A 2.8 kb *HindIII* fragment which contains the Ll.ltrB intron together with portions of the flanking exons *ltrBE1* and *ltrBE2* was recovered from the agarose gel and the single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I obtained from Gibco BRL, Gaithersburg, MD. The resulting fragment was ligated into plasmid pET-11a that had been digested with *XbaI* and treated with Klenow fragment. pET-11a was obtained from Novagen, Madison, WI.

pETLtrA19 was introduced into the *E. coli* cells using the conventional CaCl_2 -mediated transformation procedure of Sambrook et al. as described in "Molecular Cloning A Laboratory Manual", pages 1-82, 1989. Single transformed colonies were selected on plates containing Luria-Bertani (LB) medium supplemented with ampicillin to select the plasmid and with tetracycline to select the BLR strain. One or more colonies were inoculated into 2 ml of LB medium supplemented with ampicillin and grown overnight at 37°C with shaking. 1 ml of this culture was inoculated into 100 ml LB medium supplemented with ampicillin and

grown at 37° C with shaking at 200 rpm until OD₅₉₅ of the culture reached 0.4. Then isopropyl-beta-D-thiogalactoside was added to the culture to a final concentration of 1 mM and incubation was continued for 3 hours. Then the entire culture was harvested by centrifugation at 2,200 x g, 4°C, for 5 minutes. The bacterial pellet was washed with 150 mM NaCl and finally resuspended in 1/20 volume of the original culture in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (Buffer A). Bacteria were frozen at -70°C.

To produce a lysate the bacteria were thawed and frozen at -70°C three times. Then 4 volumes of 500 mM KCl, 50 mM CaCl₂, 25 mM Tris, pH 7.5, and 5 mM DTT (HKCTD) were added to the lysate and the mixture was sonicated until no longer viscous, i.e. for 5 seconds or longer. The lysate was fractionated into a soluble fraction and insoluble fraction by centrifugation at 14,000 x g, 4°C, for 15 minutes. Then 5 ml of the resulting supernatant, i.e., the soluble fraction, were loaded onto a sucrose cushion of 1.85 M sucrose in HKCTD and centrifuged for 17 hours at 4°C, 50,000 rpm in a Ti 50 rotor from Beckman. The pellet which contains the RNP particles was washed with 1 ml water and then dissolved in 25 µl 10 mM Tris, pH 8.0, 1 mM DTT on ice. Insoluble material was removed by centrifugation at 15,000 x g, 4°C, for 5 minutes. The yield of RNP particles prepared according to this method comprise the excised Ll.ltrB intron RNA and the *ltrA* protein.

Preparation of Substrate DNA

Labeled DNA substrates having sequences from the E2/E3 junction of the yeast mitochondrial *COX I* gene, the E1/E2 junction of the yeast mitochondrial *COX I* gene, and the E1/E2 junction of the putative *Lactococcus lactis* relaxase gene (*ltrB*) were synthesized from recombinant plasmids or synthetic oligonucleotide templates by PCR or primer extension. The sequence of the substrate containing the E2/E3 junction of the yeast mitochondrial *COX I* gene is depicted in Figure 3. The sequence, SEQ ID NO ____ of the top strand of such substrate is denoted as wt. The sequence of the substrate containing the E1/E2 junction of the yeast mitochondrial *COX I* gene is depicted in Figure 6, which also identifies the locations of the mutations in this sequence. . The sequence, SEQ ID NO ____ of the top strand of such substrate is denoted as wt in Figure 6. The sequence of the substrate containing the E1/E2 junction of the putative *Lactococcus lactis* relaxase gene (*ltrB*) is depicted in Figure 8, which also identifies the locations of the mutations in this sequence. The sequence of the top strand of a DNA substrate containing this E1/E2 junction is denoted as wt in Figure 8. DNA substrates that were labeled on the 5' end of the antisense strand were also generated from plasmids by PCR with 200 ng of the 5' end-labeled primer and

unlabeled primer, both of which are complementary to a sequence in the polylinker. Single-stranded DNA substrates were synthesized by end-labeling nucleotides. Short segments of double-stranded DNA substrates were also prepared

The following examples of methods employing nucleotide integrases comprising an excised aI2 intron RNA bound to an aI2 protein, an excised aI1 intron RNA bound to an aI1 protein, or an excised ltrA intron RNA bound to an ltrA protein to cleave DNA substrates are for illustration only and are not intended to limit the scope of the invention.

Example 1 Cleaving a Double-Stranded DNA Substrate with a Nucleotide Integrase

Comprising a wild-type aI2 Intron RNA and a wild-type aI2-Encoded Protein

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with a DNA substrate consisting of yeast mitochondrial *COX1* exons 2 and 3 (E2E3) and comprising the WT sequence shown in Figure 3. The reaction was conducted at 37°C in a buffer containing 100 mM KCl, 20 mM MgCl₂ at pH7.5. One portion of the cleavage products was denatured with glyoxal and analyzed in a 1% agarose gel to determine the extent of cleavage of the top strand or sense strand of the DNA substrate at the E2/E3 junction. Another portion of the nucleic acid cleavage products was analyzed in a denaturing 6% polyacrylamide gel to determine the extent of cleavage of both strands of the double stranded DNA substrate. The gels were dried and autoradiographed or quantitated by phosphorimaging with a Molecular Dynamics Phosphorimager 445.

The results indicated that the nucleotide integrase comprising an excised aI2 intron RNA from wild-type yeast bound to an aI2 intron-encoded protein from wild-type yeast cleaved the top strand of a substrate having the wt target sequence at the position marked by the arrowhead in Figure 3. The results also indicated that the group II intron RNA is integrated into the cleavage site of the sense strand. The results also indicated that the nucleotide integrase cleaved the bottom strand or antisense strand of the double-stranded DNA substrate at a location 10 base pairs downstream from the cleavage site in the first strand.

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with six different derivatives of the wt DNA substrate of Figure 3. Each of the derivatives contained a single point mutation in IBS2 of the wt sequence shown in Figure 3. In the derivatives, the nucleotides in the -7, -8, -9, -10, -11, -12, and -13 were each changed to its complement. The reactions were conducted and the cleavage products assayed on a 1% agarose gel as described above. The results indicated that the ability of this nucleotide integrase to cleave a

double-stranded DNA substrate was reduced unless there was full complementarity between each of the nucleotides of EBS2 of the α 12 intron RNA and each of the nucleotides of the IBS2 of the substrate. The only exception occurred with the substrate having a mutation at the nucleotide at +7.

5 0.025 O.D.₂₆₀ units of the nucleotide integrase of formulation 1 were reacted with derivatives of the wt DNA substrate of Figure 3 in which the nucleotides at each of the positions from -14 to -21 in the wt sequence were separately changed to a mixture of the incorrect nucleotides. Thus, the nucleotide integrase was reacted with 10 different substrates, each of which contained a mixture of three mutations at a single site. The reactions were
10 conducted as described above in example 1 and the cleavage products were glyoxylated and assayed on a 1% agarose gel. The results indicated that the nucleotide integrase cleaved substrates having point mutations at position -21, -20, -17, and -14 in the target sequence at levels that ranged from 67% to 115% of the levels achieved when the nucleotide integrase was reacted with the wt sequence depicted in Figure 3. The levels of cleavage were reduced
15 to the greatest extent with the substrates having point mutations at -15 and -18. The level of cleavage that occurred with substrates having mutations at -15 and -18 was 9% and 3% of the cleavage obtained when the nucleotide integrase was reacted with the wt sequence depicted in Figure 3. Mutations at positions -16 and -19 had moderate effects, and substrates containing these mutation were cleaved by the nucleotide integrase at levels that were 23%
20 and 31% of the levels achieved with a substrate having the wt sequence.

 0.025 O.D.₂₆₀ units of the nucleotide integrase of formulation 1 were reacted with derivatives of the DNA substrate of Figure 3 in which the nucleotides at each of the positions from +1 to +10 in the wt sequence were separately changed to a mixture of three different bases. Thus, the nucleotide integrase was reacted with 30 different substrates, each
25 of which had a mixture of the three different nucleotides. The reactions were conducted as described above in example 1 and the cleavage products were assayed on a 6% polyacrylamide gel to determine whether the nucleotides at these positions are required for cleavage of the antisense strand of the substrate containing the wt sequence. The cleavage products were also glyoxylated and analyzed on a 1% agarose gel to determine if changes in
30 the nucleotides at these positions had any effect on the ability of the nucleotide integrase to cleave the bottom or antisense strand of the substrate. The results indicated that the α 12 nucleotide integrase cleaved the bottom strand of substrates having changes at position +1, +4, and +6 in the wt sequence shown in Figure 3 at levels that were 39, 33, and 29 %, respectively of the levels achieved when the nucleotide integrase was reacted with the wt

sequence. Changes in the nucleotides at the other positions, i.e., +2, +3, +5, +7, +8, +9, and +10 had little effect on the ability of the nucleotide sequence to cleave the bottom strand of the substrate. The results also indicated that changes in the nucleotides at each of these positions had little effect on the ability of the nucleotide integrase to cleave the top strand of the mutated substrate.

Comparative Example A

0.025 O.D.₂₆₀ units of the RNP particle preparations of formulations 1, 2, 4, 5 were reacted for 20 minutes with 125 fmoles (150,000 cpm) of an internally-labeled DNA substrate having the wt sequence depicted in Figure 3. To verify cleavage, the products were glyoxalated and analyzed in a 1% agarose gel. The results indicated that nucleotide integrases which lack excised aI2 intron RNA or in which the intron-encoded protein lacks the nonconserved portion of the Zn domain, will neither cleave the double-stranded DNA substrate nor attach an RNA.

Example 2 Cleaving a Double-stranded DNA substrate with the Reconstituted RNP Particle

Preparation of Formulation 12

The reconstituted RNP particle preparation of formulation 12 was reacted with 250 fmoles (300,000 cpm) of the 142 base pair DNA substrates generated from pE2E3 and which were 5' end-labeled on either the sense strand or the antisense strand for 20 minutes at 37°C. To verify cleavage of both strands of the substrate, the reaction products were extracted with phenol-CIA in the presence of 0.3 M NaOAc and 2 mg single-stranded salmon sperm DNA followed by precipitation with ethanol. DNA reactions products were analyzed in a 6% polyacrylamide/8 M urea gel. The reconstituted particle preparation cleaved both strands of a double-stranded DNA substrate which contained the wild-type sequence shown in Figure 4. Similar results, i.e. cleavage of both strands, were obtained when the 5' end labeled substrates were incubated with the RNP particle preparation of formulation 10.

Example 3 Cleaving Double-stranded DNA Substrates with a Nucleotide Integrase Comprising a Modified aI2 Intron RNA and an aI2-Encoded Protein.

0.025 O.D.₂₆₀ units of the RNP particles of formulation 13 in which the EBS1 of the aI2 group II intron RNA was changed to the EBS1 sequence of the aI1 intron RNA was reacted with the wt DNA substrate of Figure 3 and with a derivative thereof in which the nucleotides at position -1 to -6 were changed to 5'TTAATG, which is the IBS1 sequence of the wt sequence for the aI1 nucleotide integrase. Each of the derivatives contained a single mutation in the wt sequence shown in Figure 3. The reactions were conducted and the cleavage products analyzed as described in example 1. The aI2 nucleotide integrase

comprising a group II intron RNA with a modified EBS1 was not able to cleave a substrate with the wt sequence but was able to cleave a substrate in which the nucleotides at position -1 to -6 were complementary to the modified EBS1.

Example 4 Cleaving Substrate with a Nucleotide Integrase Comprising a Wild-type or Modified aI2 Intron RNA and an aI2-Encoded Protein

0.025 O.D.₂₆₀ units of the RNP particles of formulation 1 were reacted with three different derivatives of the DNA wt substrate of Figure 3. Each of the derivatives contained a single point mutation. In the derivatives the nucleotide at +1 was changed to either a C, G, or A. The derivatives were also reacted with a nucleotide integrase comprising an aI2 intron RNA in which the nucleotide immediately preceding EBS1 was either an A, G, C, or T. The reactions were conducted and the cleavage products assayed on a 1% agarose gel as described in example 1. The results indicated that cleavage of the top strand is enhanced when the nucleotide at +1 is complementary to the nucleotide immediately preceding the EBS1 in the aI2 intron RNA and that cleavage of the sense strand is strongly reduced when the target sequence has a G at the +1 position.

Example 5 Cleaving Double-Stranded DNA Substrates with a Nucleotide Integrase Comprising an aI1 intron RNA and an aI1 intron-encoded protein.

Double-stranded DNA substrates comprising either the wt sequence or an altered sequence having one of the eleven single point mutations depicted in Figure 6 were reacted with the RNP particle preparation of Formulation 3. For each reaction, 1.5 nM (about 150000 cpm) of a double-stranded DNA substrate was mixed with 0.025 OD₂₆₀ units of the RNP particle preparation in 10 µl of 50 mM Tris pH 7.5, 5 mM KCl, 10 mM MgCl₂, 5mM DTT. The reaction mixtures were incubated for 20 minutes at 37°C. The reaction was stopped by adding 70 µl of 0.5 mM EDTA, 0.15 mg/ml tRNA. The nucleic acids were phenol extracted, ethanol precipitated, glyoxylated and analyzed on a 1% agarose gel.

The nucleotide integrase of formulation 3 cleaved substrate DNAs having mutations at positions -23, -20, -17, -16, -15 and -14 as efficiently as a substrate having the wt sequence depicted in Figure 6. Mutations at positions G(-22), G(-21), A(-19) and A(-18) reduced the efficiency of the cleavage somewhat from 75 to 25% of the cleavage that occurred with the wt sequence. The most critical nucleotide appears to be the C at position(-13). Mutations at this position reduced cleavage of the substrate to less than 1% of that which occurred with the wt sequence.

Mutations in the downstream region +1 to +10 had variable effects on cleavage of the

top strand. Substitution of the nucleotide at +1 which disrupts the δ - δ' interaction strongly inhibited cleavage. The substitution at +2 inhibited cleavage of the top strand by about 50%, whereas other substitutions had relatively little effect.

The ability of the nucleotide integrase of formulation 3 to cleave the other or the bottom strand of substrates was also determined. Substitutions at positions -13, -18, -19, and -21 reduced cleavage of both the bottom strand and the top strand of the double-stranded substrate. In the downstream region, nucleotide substitutions at +4 and +6 strongly inhibited cleavage of the bottom strand. (9 and 1% activity, respectively). Nucleotide substitutions at +7 and +9 inhibited moderately (53% and 18% activity, respectively), while nucleotide substitutions at +2, +3, +5, and +10 reproducibly stimulated cleavage of the bottom strand.

Example 6 Cleaving substrates with a Nucleotide Integrase Comprising a wild-type or modified L1.ltrB intron RNA and an ltrA protein.

Double-stranded DNA substrates comprising either the wt sequence or an altered wt sequence having one of the eleven single point mutations depicted in Figure 8 were reacted with the RNP particle preparation of Formulation 15. The point mutations occur at positions -23 to -13 in the wt sequence. For each reaction, 1.5 nM of a double-stranded DNA substrate was mixed with 0.025 OD₂₆₀ units of the RNP particle preparation in 10 μ l of 50 mM Tris pH 7.5, 10 mM KCl, 10 mM MgCl₂, 5mM DTT. The reaction mixtures were incubated for 20 minutes at 37°C. The reaction was stopped by adding 70 μ l of 28.6 mM EDTA, 0.15 mg/ml tRNA. The nucleic acids were phenol extracted, ethanol precipitated, glyoxylated and analyzed on a 1% agarose gel

The nucleotide integrase of formulation 15 cleaved substrate DNAs having mutations at positions C(-22), C(-18), and A(-14) at levels that were approximately 80% of the levels achieved with a substrate having the wt sequence depicted in Figure 8. Substrates having point mutations at positions G(-21), A(-20), T(-19) were cleaved at levels that were approximately 40% or less of the levels achieved using substrates having a wt sequence.

The nucleotide integrase of formulation 15 was also reacted with double stranded DNA substrates in which the IBS1 and IBS2 sequences were modified as shown in Fig. 12. First, a DNA substrate in which both the IBS1 sequence (-1 to -7) and the IBS2 sequence (-8 to -13) were changed to their complementary sequence (cIBS1IBS2) gave less than 10% of the activity of the wild-type substrate. Next, each position in the IBS1 sequence and the IBS2 sequence as well as the δ' nucleotide (-13 to +1) were modified by changing A or G residues to a mixture of pyrimidine residues, and C or T residues to a mixture of purine residues.

Substrates having substitutions at positions -12, -11, -10, -6, -4, and +1 were cleaved by the wild-type ltrA nucleotide integrase at levels that were 10 to 30% of the levels achieved when the substrate contained the wild-type sequence shown in Fig. 12. The ltrA nucleotide integrase cleaved substrates having substitutions at position -13, -9, -8, -2, and -1 at levels that were about 60% of the wild-type activity. Substitutions at -7, -5, and -3 in the substrate sequence had little to no effect on the ability of the ltrA nucleotide integrase to cleave the top strand of the substrate (92 to 118% of wild-type activity). Further, a DNA substrate in which position -7, -5, and -3 were changed simultaneously gave about 70% of wild-type activity, consistent with the relatively small effects of single nucleotide substitutions at these positions. The results indicate that base pairing between the nucleotides in the EBS 1 sequence of the Ll.ltrB intron RNA and the nucleotides at positions -3, -5, and -7 is not required for cleavage of the top strand of a double-stranded DNA substrate by the ltrA nucleotide integrase.

The ltrA nucleotide integrase comprising a wild-type Ll.ltrB intron RNA and an ltrA protein was also reacted with double stranded DNA substrates in which the nucleotides at positions +2 to +19, +21, to +24, +30 and +32 of the wild-type sequence shown in Fig. 12 were simultaneously changed to their respective complement. The ltrA integrase cleaved substrates having multiple substitutions in these downstream nucleotides at levels that were 70% of those achieved with the wild-type substrate.

An ltrA nucleotide integrase comprising a wild-type Ll.ltrB intron RNA and an ltrA protein was also reacted with a double stranded DNA substrate in which the A-T base-pairs at positions -26 and -23 of the wild-type sequence shown in Fig. 12 were changed to G-C base-pairs, and with a double-stranded DNA substrate in which the A-T base-pairs at positions -16 and -14 were changed to G-C base pairs. The wild-type ltrA nucleotide integrase cleaved the modified substrates at levels that were about 35% and 65%, respectively of the levels achieved with the wild-type substrate. Thus, except for those nucleotide pairs that are at critical positions in the first sequence element (such as for example the G-C pair at position - 21), it is preferred that the first sequence element be A-T rich and G-C poor.

An ltrA nucleotide integrase comprising a wild-type Ll.ltrB intron RNA and an ltrA protein was also reacted with double stranded DNA substrates in which the sequence of nucleotides at positions +2 to +5 of the wild-type sequence shown in Fig. 12 was changed to the more G/C rich sequence TGCG. The wild-type ltrA nucleotide integrase cleaved the modified substrates at levels that were less than 10% of the levels achieved with the wild-type substrate. Thus, it is preferred that the second sequence element, particularly the first five pairs of nucleotides downstream of the cleavage site, be A-T rich or G-C poor.

LtrA nucleotide integrases containing a modified Ll.ltrB intron RNA and an ltrA protein were reconstituted by using purified LtrA protein and *in vitro* synthesized intron RNA. The modifications were made in the EBS1 nucleotide that binds to the nucleotide at position -4 in the substrate and in the nucleotides of the EBS2 sequence that bind to the nucleotides at positions -10, -11, and -12 in the substrate. Modifications were also made in the δ nucleotide. Cleavage of the top strand of substrates in which the nucleotides at positions -10, -11 and +1 were changed from wild-type was restored by compensatory mutations in the corresponding EBS2 positions and the δ nucleotide of the intron RNA. A compensatory change in the EBS2 nucleotide that binds to the nucleotide at -12 in the substrate partially restored the cleavage activity of the nucleotide integrase, while a nucleotide integrase comprising a compensatory change in the EBS1 nucleotide that binds to the nucleotide at -4 in the substrate exhibited no detectable cleavage activity. Thus, it is preferred that the ltrA nucleotide integrase be used to cleave double-stranded DNA substrates that have a T at position -4 relative to the cleavage site.

Example 7 Cleaving a Double-Stranded DNA Substrate with Purified RNP Particles

125 fmoles (150,000 cpm) of an internally-labeled substrate containing of yeast mitochondrial *COX1* exons 2 and 3 (E2E3) and comprising the WT sequence shown in Figure 3 were incubated with 10 μ l of each of the fractions obtained from the sucrose gradient in formulation 1a. Taking into account the composition of the fractions, the final reaction medium of 20 μ l contained 100 mM KCl, 20 mM MgCl₂, 50 mM Tris-HCl, pH 7.5, and 5 mM DTT. Following a 20 minute reaction at 37°C, 30 μ l of water, 5 μ l 0.3 M NaOAc and 5 μ g tRNA were added to the fractions. The reaction products were phenol extracted, ethanol precipitated, glyoxalated, separated on a 1% agarose gel and analyzed by autoradiography of the dried gel. The results indicated that the purified RNP particles of formulation 1a are useful to cleave both strands of a double-stranded DNA substrate and to insert the α 2 intron RNA into the cleavage site.

Example 8 Cleaving Both Strands of a Double-stranded DNA Substrate and Attaching a cDNA to the Cleavage Site of the Bottom Strand.

0.025 O.D.₂₆₀ units of the RNP particles from formulations 1,2,4,5,6,7,8,9, were incubated with 250 fmoles (300,000 cpm) of a 142 base pair DNA substrate comprising the WT sequence shown in Figure 3. DNA incubation products were analyzed in a 6% polyacrylamide/8 M urea gel.

A radiolabeled band corresponding to the 5' fragment was detected when RNP particles of formulations 1 and 2 were incubated with substrates that had been labeled on the

5' end of either the top strand or the bottom strand of the DNA substrate, indicating that these particles cleaved both strands of the DNA substrate. The RNP particles of formulation 1 cleaved the top strand precisely at the exon 2-exon 3 junction. The RNP particles of formulations 1 and 2 cleaved the bottom or antisense strand 10 base pairs downstream from the top or sense strand cleavage site. RNP particles of formulation 1 that had been treated with protease K, or RNase A, or boiled did not cleave either strand.

Radiolabeled bands were also detected when the RNP particles of formulation 4 were incubated with DNA substrates that had been 5' end-labeled on either the sense strand or antisense strand, indicating that this nucleotide integrase cleaved both strands of DNA substrate. The RNP particles of formulation 4 contain a modified, excised aI2 RNA and an aI2-encoded protein which lacks detectable reverse transcriptase activity. Although the extent of cleavage of RNP particles of formulation 4 is somewhat reduced compared to cleavage with the RNP particle preparation of formulation 1, the endonuclease activity of the RNA is present even when the reverse transcriptase activity of the aI2-encoded protein is absent.

The radiolabeled bands were detected when the RNP particles of formulation 5 were incubated with the DNA substrate that had been labeled on the 5' end of either the top or bottom strand. In quantitative assays normalized by either O.D.₂₆₀ or soluble aI2 reverse transcriptase activity, the cleavage activities for the top and bottom strands by the RNP particles of formulation 5 were 6% and 25%, respectively, of activities of the RNP particles of formulation 1.

A radiolabeled band corresponding to the 5' fragment was detected when the DNA substrate labeled on the 5' end of the top strand was incubated with the RNP particles of formulation 6, but a band corresponding to the 5' fragment of the top strand was not detected when the RNP particles of formulation 6 were incubated with a DNA substrate that had been labeled on the 5' end of the bottom strand. The RNP particles of formulation 6 contain a modified, excised aI2 intron RNA and an aI2-encoded protein that has an alteration in one of the putative endonuclease motifs. Similar results were obtained with the RNP particles of formulation 7, which contains a modified, excised aI2 intron RNA and an aI2-encoded protein in which the conserved portion of the Zn domain is absent. Likewise, RNP particles of formulations 8 and 9, each of which contains a modified, excised aI2 intron RNA and an aI2-encoded protein in which there is a mutation in the Zn²⁺-like motif, cleaved the sense strand but not the antisense strand of the DNA substrate. For the RNP particles of formulations 6, 7, 8, and 9, the level of sense-strand cleavage was proportional to the amount

of RNA-DNA products detected in the agarose gels. These findings indicate that the antisense strand endonuclease activity of the aI2-encoded protein is associated with the Zn domain.

A radiolabeled band corresponding to the 5' fragment was detected when the reconstituted RNP particle preparation of formulation 12 was incubated with substrates that had been labeled on the 5' end of either the sense strand or the antisense strand of the DNA substrate. These results establish that the reconstituted RNP particle preparation cleaves both strands of the DNA substrate.

Thus, both the catalytic RNA molecule of the nucleotide integrase and the intron-encoded protein are required for cleavage of both strands of the double stranded DNA. Certain modifications in the Zn domain and the X domain of intron-encoded protein disrupt the cleavage of the antisense strand of the nucleotide integrase

0.025 O.D.₂₆₀ units of the RNP particle preparations of formulations 1, 2, 4 and 5 were combined in 10 µl of reaction medium with 1 µg of plasmid containing the wild-type sequence depicted in Figure 4. The reaction medium contained 0.2 mM each of dATP, dGTP and dTTP, 10 µCi [α -³²P]-dCTP (3,000 Ci/mmmole; DuPont NEN, Boston, MA), 100 mM KCl, and 5 mM dithiothreitol, 2 mM MgCl₂, and 50 mM Tris-HCl, pH 8.5. The reaction was initiated by addition of the RNP preparations, incubated for 10 minutes at 37°C, and chased with 0.2 mM dCTP for another 10 minutes. After the chase period, the reactions were terminated by extraction with phenol-CIA (phenol-chloroform-isoamyl alcohol; 25:24:1) in the presence of 0.3 M sodium acetate, pH 7.8, and 5 µg E. coli tRNA carrier (Sigma, St. Louis, MO). Products were ethanol precipitated twice and resolved in 1% agarose gels containing 90 mM Tris-borate, pH 8.3, 2 mM EDTA and 0.05% ethidium bromide. The results indicated that the RNP particles of formulations 1 and 2 catalyze the formation of a DNA molecule on the cleaved DNA substrate. The results also indicated that a nucleotide integrase which lacks an excised group II intron RNA or which contains a group II intron-encoded protein that lacks a reverse transcriptase domain does not catalyze the formation of a cDNA molecule on the cleaved strand.

Cleavage of single stranded DNA

An aI2 nucleotide integrase comprising an excised aI2 RNA and aI2-encoded protein was used to cleave a single stranded DNA comprising an IBS2 and IBS1 sequence complementary to the EBS1 and EBS2 sequences of the wild-type aI2 intron RNA. The reaction is greatly improved when the 3 nucleotides +1 to +3 can base-pair with the 3 nucleotides immediately upstream of EBS1. The most preferred reaction conditions for

cleavage of the substrate and insertion of the intron RNA into the cleavage site by the nucleotide integrase, are 100 mM KCl, 20 mM MgCl₂, pH 7.5, 5 mM DTT and 37°C.

CLAIMS

What is claimed is:

- 1 1. A method of cleaving a double stranded DNA substrate at a cleavage site comprising the
2 following steps:
 - 3 (a) providing a double-stranded DNA substrate having a top strand and a recognition
4 site; wherein said top strand has a T at position -4 relative to the cleavage site;
 - 5 (b) providing an isolated nucleotide integrase comprising
 - 6 (i) a wild-type or a modified Ll.ltrB intron RNA having a first hybridization
7 sequence for hybridizing with a first intron RNA binding sequence on said top
8 strand of the DNA substrate and a second hybridization sequence for
9 hybridizing with a second RNA binding sequence on said top strand of the
10 substrate; and
 - 11 (ii) a protein encoded by an Ll.ltrB intron for binding with at least one
12 nucleotide in a first sequence element in the recognition site of the substrate,
13 said protein being bound to said Ll.ltrB intron RNA; and
 - 14 (c) reacting the nucleotide integrase with the substrate to permit the nucleotide
15 integrase to cleave said top strand of the DNA substrate and to insert the Ll.ltrB intron
16 RNA into the cleavage site.
- 1 2. The method of claim 1 wherein the top strand of the substrate further has a G at -21
2 and an A at -20 relative to the cleavage site and wherein the top strand of the substrate has a
3 sequence which differs from the wt sequence, SEQ. ID. NO: _____, shown in Figure 8.
- 1 3. The method of claim 2 wherein the top strand of the substrate further has a T at -19, a
2 G at -17, and a G at -15 relative to the cleavage site.
- 1 4. The method of claim 2 wherein the nucleotide at -3, or -5, or -7 on the top strand of
2 the substrate is not complementary to a nucleotide at the corresponding position in the first
3 hybridization sequence of the Ll.ltrB intron RNA.
- 1 5. The method of claim 1 wherein the sequence of nucleotides from +1 through +5 on
2 the top strand of the substrate contains more A and T nucleotides than C and G nucleotides.
- 1 6. The method of claim 1 wherein the sequence of nucleotides from -14 to -26 on the top

2 strand of the substrate contains more A and T nucleotides than C and G nucleotides.

1 7. The method of claim 1 wherein the top strand of the substrate further has a C at -12,
2 an A at -11, a C at -10, a C at -6, and a C at +1 relative to the cleavage site and wherein the
3 top strand of the substrate has a sequence which differs from the wt sequence, SEQ. ID. NO:
4 _____, shown in Figure 8.

1 8. The method of claim 1 wherein the top strand of the substrate has a nucleotide that is
2 complementary to the nucleotide on said top strand of the substrate, said nucleotide being
3 located at position +1 relative to the cleavage site and wherein the top strand of the substrate
4 has a sequence which differs from the wt sequence, SEQ. ID. NO: _____, shown in Figure 8.

1 9. The method of claim 1 wherein there is at least 80% complementarity between the
2 first hybridization sequence and the first intron RNA binding sequence and at least 80%
3 complementarity between the second hybridization sequence and the second intron RNA-
4 binding sequence and wherein the top strand of the substrate has a sequence which differs
5 from the wt sequence, SEQ. ID. NO: _____, shown in Figure 8.

1 10. A method of cleaving both strands of a double-stranded DNA substrate comprising
2 the following steps:

3 (a) providing a double stranded DNA substrate having a top strand, wherein said top
4 has a C at -13, a G at -21, an A at -19, an A at -18, a T at +4, and a G at +6 relative to
5 the cleavage site, and wherein the top strand of the substrate has a sequence which
6 differs from the wt sequence, SEQ. ID. NO: _____, shown in Figure 6;

7 (b) providing an isolated nucleotide integrase comprising;

8 (i) a wild-type or a modified a11 intron RNA having a first hybridization
9 sequence for hybridizing with a first intron RNA binding sequence on said top
10 strand of the DNA substrate and a second hybridization sequence for
11 hybridizing with a second RNA binding sequence on said top strand of the
12 substrate, a nucleotide that is complementary to a ' nucleotide on the top
13 strand of the substrate, said ' nucleotide being located at position +1 relative
14 to the cleavage site; and

15 (ii) a protein encoded by an a11 intron for binding with at least one nucleotide
16 in a first sequence element in the recognition site of the substrate, said protein

17 being bound to said group II intron RNA; and
18 (c) reacting the nucleotide integrase with the substrate to permit the nucleotide
19 integrase to cleave both strands of the DNA substrate and to insert the aI1 intron RNA into
20 the cleavage site on said top strand.

1 11. The method of claim 10 wherein there is at least 80% complementarity between the first
2 hybridization sequence and the first intron RNA binding sequence and at least 80%
3 complementarity between the second hybridization sequence and the second intron RNA-
4 binding sequence.

1 13. The method of claim 10 wherein the substrate further has a T at + 7 and a G at + 9
2 relative to the cleavage site.

1 14. The method of claim 14 wherein the aI1 encoded protein comprises a reverse
2 transcriptase domain, and wherein the aI1 nucleotide integrase and the substrate are reacted in
3 a reaction mixture comprising dATP, dGTP, dTTP, and dCTP such that a cDNA molecule is
4 formed in the cleavage site on the other strand of the DNA substrate.

1 15. A method for cleaving a single-stranded nucleic acid substrate at a cleavage site
2 comprising the following steps:

3 (a) providing an isolated nucleotide integrase comprising:

4 (i) a group II intron RNA having a first hybridizing sequence for hybridizing
5 with a first intron RNA binding sequence on the nucleic acid substrate and a
6 second hybridizing sequence for hybridizing with a second hybridizing
7 sequence on said nucleic acid substrate, wherein the three nucleotides
8 immediately upstream of the first hybridizing sequence are complementary to
9 nucleotides at positions +1 to +3 on the substrate, and

10 (ii) a group II intron-encoded protein bound to said group II intron RNA; and
11 (b) reacting the nucleotide integrase with the substrate to permit the nucleotide
12 integrase to cleave the nucleic acid substrate and to insert the group II intron RNA
13 into the cleavage site.

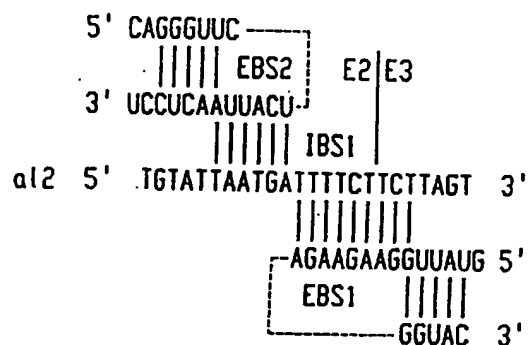


Fig. 1

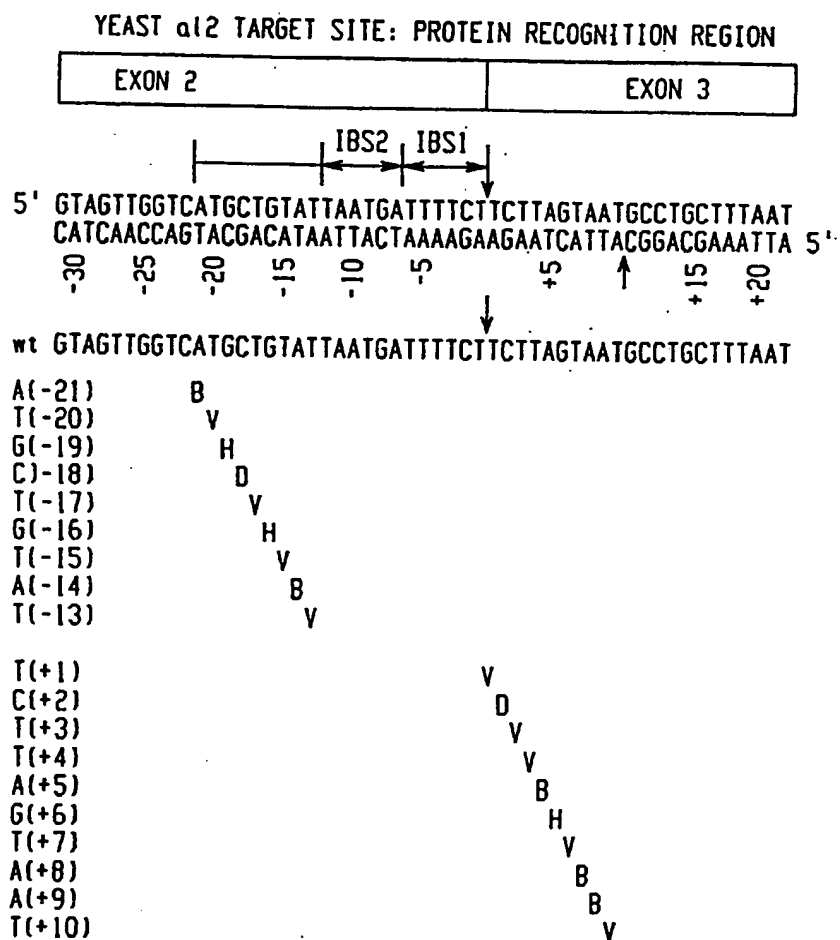


Fig. 3

S. cerevisiae 16l mt coxl E1, a11, E2, a12, E3

<E1
ATGGTACAAA GATGATTATA TTCAACAAAT GCAAAAGATA TTGCAGTATT ATATTTTATG 60
TTAGCTATTT TTAGTGGTAT GGCAGGAACA GCAATGTCTT TAATCATTAG ATTAGAATTA 120
GCTGCACCTG GTTCACAATA TTTACATGGT AATTCACAGT TATTTAATGG TGCGCCTCTC E1> a11 180
AGTGCCTATA TTTCGTTGAT GCGCTAGCA TTAGTATTAT GAATCATCAA TAGATACTTA 240
AAACATATGA CTAATCAGT AGGGGCTAAC TTTACGGGGA CAATAGCATG TCATAAAACA 300
CCTATGATTA GTGTAGGTGC AGTTAAGTGT TACATGGTTA GGTAAACGAA CTTCTTACAA 360
>EBS2< 420
GTCTTTATCA GGATTACAAT TTCTCTTAT CATTGGATA TAGTAAACA AGTTTGATTA
>EBS1< 480
TTTTACGTTG AGGTAATCAG ATTATGATTC ATTGTTTTAG ATAGCACAGG CAGTGTGAAA
AAGATGAAGG ACCTAAATAA CACAAAAGGA AATACGAAAA GTGAGGGATC AACTGAAAGA 540
GGAACTCTT GAGTTGACAG AGGTATAGTA GTACCGAATA CTCAAATAAA AATGAGATTT 600
TTAAATCAAG TTAGATACTA TTCAGTAAAT AATAATTTAA AAATAGGGAA GGATACCAAT 660
ATTGAGTTAT CAAAAGATAC AAGTACTTCG GACTTGTTAG AATTTGAGAA ATTAGTAATA 720
GATAATATAA ATGAGGAAAA TATAAATAAT AATTTATTAA GTATTATAAA AAACGTAGAT 780
ATATTAATAT TAGCATATAA TAGAATTAAG AGTAAACCTG GTAATATAAC TCCAGGTACA 840

MATCH TO FIG.2B

Fig. 2A

MATCH TO FIG. 2A

ACATTAGAAA CATTAGATGG TATAAATATA ATATATTTAA ATAAATTATC AAATGAATTA 900
GGAACAGGTA AATTCAAATT TAAACCCATG AGAATAGTTA ATATTCCTAA ACCTAAAGCT 960
GGTATAAGAC CTTTAAGTGT AGGTAATCCA AGAGATAAAA TTGTACAAGA AGTTATAAGA 1020
ATAATTTTAG ATACAATTTT TGATAAAAAG ATATCAACAC ATTCACATGG TTTTAGAAAG 1080
AATATAAGTT GTCAAACAGC AATTGAGAA GTTAGAAATA TATTGGTG6 AAGTAATTGA 1140
TTTATTGAAG TAGACTTAAA AAAATGTTTT GATACAATTT CTCATGATTT AATTATTAA 1200
GAATTAAAAA GATATATTTT AGATAAAGGT TTTATTGATT TAGTATATAA ATTATTAAGA 1260
GCTGGTTATA TTGATGAGAA AGGAACTTAT CATAAACCTA TATTAGGTTT ACCTCAAGGA 1320
TCATTAATTA GTCCTATCTT ATGTAATATT GTAATAACAT TGGTAGATAA TTGATTAGAA 1380
GATTATATTA ATTTATATAA TAAAGGTAAA GTTAAAAAAC AACATCCTAC ATATAAAAAA 1440
TTATCAAGAA TAATTGCAAA AGCTAAAATA TTTTCGACAA GATTAAAATT ACATAAAGAA 1500
AGAGCTAAAG GCCCACTATT TATTTATAAT GATCCTAATT TCAAGAGAAT AAAATACGTT 1560
AGATATGCAG ATGATATTTT AATTGGGGTA TTAGGTTCAA AAAATGATTG TAAAATAATC 1620
AAAAGAGATT TAAACAATTT TTAAATTCA TTAGGTTTAA CTATAAATGA AGAAAAAACT 1680

MATCH TO FIG. 2C*Fig. 2B*

MATCH TO FIG. 2B

1740
TTAATTACTT GTGCAACTGA ACTACCAGCA AGATTTT TAG GTTATAATAT TTCAATTACA
1800
CCTTTAAAA GAATACCTAC AGTTACTAAA CTAATTAGAG GTAACTTAT TAGAAGTAGA
1860
AATACAATA GACCTATTAT TAATGCACCA ATTAGAGATA TTATCAATAA ATTAGCTACT
1920
AATGGATATT GTAAGCATAA TAAAAATGGT AGAATAGGAG TGCCTACAAG AGTAGGTAGA
1980
TGACTATATG AGAACCTAG AACAATTATT AATAATTATA AAGCGTTAGG TAGAGGTATC
2040
TTAAATTATT ATAAATTAGC TACTAATTAT AAAAGATTAA GAGAAAGAAT CTATTACGTA
2100
TTATATTATT CATGTGTATT AACTTTAGCT AGTAAATATA GATTAAAAAC ATTAAGTAAA
2160
ACTATTAAAA AATTGGTTA TAATTTAAAT ATTATTGAAA ATGATAAATT AATTGCCAAT
2220
TTTCCAAGAA ATACTTTTGA TAATATCAAA AAAATTGAAA ATCATGGTAT ATTTATATAT
2280
ATATCAGAAG CTAAAGTAAC TGATCCTTTT GAATATATCG ATTCAATTAA ATATATATTA
2340
CCTACAGCTA AAGCTAATTT TAATAAACCT TGTAGTATTT GTAATTCAAC TATTGATGTA
2400
GAAATACATC ATGTAAACA ATTACATAGA GGTATATTAA AAGCACTTAA AGATTATATT
2460
CTAGGTAGAA TAATTACCAT AAACAGAAAA CAAATTCCAT TATGTAAACA ATGTCATATT
2520
AAAACACATA AAAATAAATT TAAAAATATA GCACCTGGTA TATAAAATCT ATTATTAATG

MATCH TO FIG. 2D

Fig. 2C

MATCH TO FIG. 2C

2580
 ATACTCAATA TGGAAAGCCG TATGATGGGA AACTATCACG TACGGTTTGG GAAAGGCTCT
 2640
 TTAACACGTG GCAACATAGG TTAATTGCT $\alpha 11 > E2$ ATTACATTT TAGTAGTTGG TCATGCTGTA
 2700
 TTAATGATTT $E2 > \alpha 12$ TCTGTGCGCC GTTTCGCTTA ATTTATCACT GTATTGAAGT GTTAATTGAT
 2760
 AAACATATCT CTGTTTATTC AATTAATGAA AACTTTACCG TATCATTTTG GTTCTGATTA
 2820
 TTAGTAGTAA CACATAGT ATTTAGATAC GTAAACCATA TGGCTTACCC AGTTGGGGCC
 2880
 AACTCAACGG GGACAATAGC ATGCCATAAA AGCGCTGGAG TAAAACAGCC AGCGCAAGGT
 > EBS2 <
 AAGAACTGTC CGATGGCTAG GTTAACGAAT TCCTGTAAAG AATGTTTAGG GTTCTCATT
 > EBS1 < 3000
 ACTCCTTCCC ACTTGGGGAT TGTGATTCAT GCTTATGTAT TGAAGAAGA GGTACAGAG
 3060
 TTAACCAAAA ATGAATCATT AGCTTTAAGT AAAAGTTGAC ATTTGGAGGG CTGTACGAGT
 3120
 TCAAATGGAA AATTAAGAAA TACGGGATTG TCCGAAAGGG GAAACCCTGS GGATAACGGA
 3180
 GTCTTCATAG TACCCAAATT TAATTTAAAT AAAGCGAGAT ACTTTAGTAC TTTATCTAAA
 3240
 TTAAATGCAA GGAAGGAAGA CAGTTTAGCG TATTTAACA AGATTAATAC TACGGATTTT
 3300
 TCCGAGTTAA ATAAATTAAT AGAAAATAAT CATAATAAAC TTGAAACCAT TAATACTAGA
 3360
 ATTTTAAAT TAATGTCAGA TATTAGAATG TTATTAATTG CTTATAATAA AATTAAAAGT

MATCH TO FIG. 2E

Fig. 2D

MATCH TO FIG. 2D

AAGAAAGGTA ATATATCTAA AGGTTCTAAT AATATTACCT TAGATGGGAT TAATATTTCA 3420
TATTTAAATA AATTATCTAA AGATATTAAC ACTAATAYGT TTAAATTTTC TCCGGTTAGA 3480
AGAGTTGAAA TTCCTAAAAC ATCTGGAGGA TTTAGACCTT TAAGTGTGG AAATCCTAGA 3540
GAAAAAATTG TACAAGAAAG TATGAGAATA ATATTAGAAA TTATCTATAA TAATAGTTTC 3600
TCTTATTATT CTCATGGATT TAGACCTAAC TTATCTTGTT TAACAGCTAT TATTCAATGT 3660
AAAAATTATA TGCAATACTG TAATTGATTT ATTAAAGTAG ATTTAAATAA ATGCTTTGAT 3720
ACAATTCCAC ATAATATGTT AATTAATGTA TTAAATGAGA GAATCAAAGA TAAAGGTTTC 3780
ATAGACTTAT TATATAAATT ATTAAGAGCT GGATATGTTG ATAAAAATAA TAATTATCAT 3840
AATACAACCT TAGGAATTCC TCAAGGTAGT GTTGTCAGTC CTATTTTATG TAATATTTT 3900
TTAGATAAAT TAGATAAATA TTTAGAAAAT AAATTTGAGA ATGAATTCAA TACTGGAAAT 3960
ATGTCTAATA GAGGTAGAAA TCCAATTTAT AATAGTTTAT CATCTAAAT TTATAGATGT 4020
AAATTATTAT CTGAAAAATT AAAATTGATT AGATTAAGAG ACCATTACCA AAGAAATATG 4080
GGATCTGATA AAAGTTTAA AAGAGCTTAT TTTGTTAGAT ATGCTGATGA TATTATCATT 4140
GGTGTAAATGG GTTCTCATAA TGATTGTAAA AATATTTTAA ACGATATTAA TAACCTCTTA 4200

MATCH TO FIG. 2F*Fig. 2E*

MATCH TO FIG. 2E

AAAGAAAATT TAGGTATGTC AATTAATATA GATAAATCCG TTATTAAACA TTCTAAAGAA 4260
GGAGTTAGTT TTTTAGGGTA TGATGTAAAA GTTACACCTT GAGAAAAAAG ACCTTATAGA 4320
ATGATTAAAA AAGGTGATAA TTTTATTAGG GTTAGACATC ATACTAGTTT AGTTGTTAAT 4380
GCCCCATTA GAAGTATTGT AATAAAATTA AATAAACATG GCTATTGTTC TCATGGTATT 4440
TTAGGAAAAC CCAGAGGGGT TGGAAGATTA ATTCATGAAG AAATGAAAAC CATTTTAATG 4500
CATTACTTAG CTGTTGGTAG AGGTATTATA AACTATTATA GATTAGCTAC CAATTTTACC 4560
ACATTAAGAG GTAGAATTAC ATACATTTTA TTTTATTCAT GTTGTTTAAC ATTAGCAAGA 4620
AAATTTAAAT TAAATACTGT TAAGAAAGTT ATTTTAAAT TCGGTAAAGT ATTAGTTGAT 4680
CCTCATTCAA AAGTTAGTTT TAGTATTGAT GATTTTAAAA TTAGACATAA AATAAATATA 4740
ACTGATTCTA ATTATACACC TGATGAAATT TTAGATAGAT ATAAATATAT GTTACCTAGA 4800
TCTTTATCAT TATTTAGTGG TATTTGTCAA ATTTGTGGTT CTAAACATGA TTTAGAAGTA 4860
CATCACGTAA GAACATTAAA TAATGCTGCC AATAAAATTA AAGATGATTA TTTATTAGGT 4920
AGAATGATTA AGATAAATAG AAAACAAATT ACTATCTGTA AAACATGTCA TTTTAAAGTT 4980
CATCAAGGTA AATATAATGG TCCAGGTTTA TAATAATTAT TATACTCCTT CGGGGTCGCC 5040

MATCH TO FIG. 2G*Fig. 2F*

MATCH TO FIG. 2F

GC GGGGGCGG GCCGGACTAT TAAATATGCG TTAAATGGAG AGCCGTATGA TATGAAAGTA 5100

TCACGTACGG TTCGGAGAGG GCTCTTTTAT ATGAATGTTA TTACATTCAG ATAGGTTTGC 5160

$\alpha 12 > E3$
TACTCTACTC TTAGTAATGC CTGCTTTAAT TGGAGGTTTT GGT E3

Fig. 2G

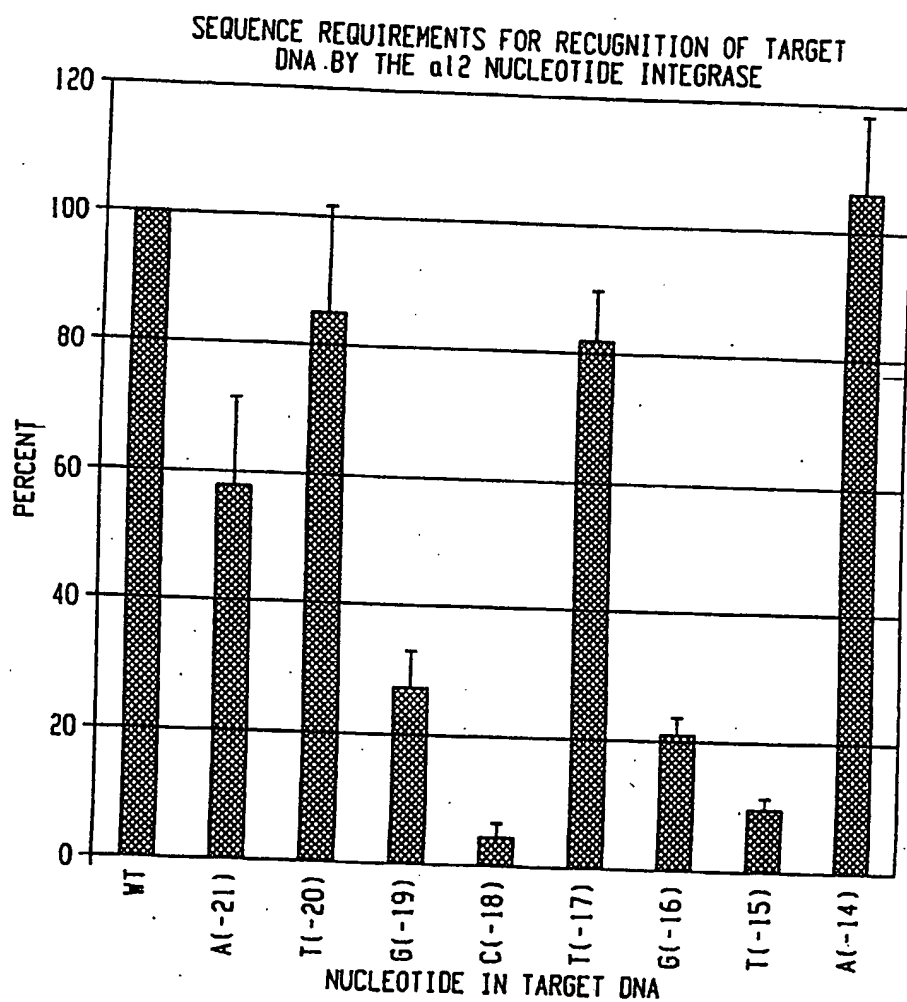
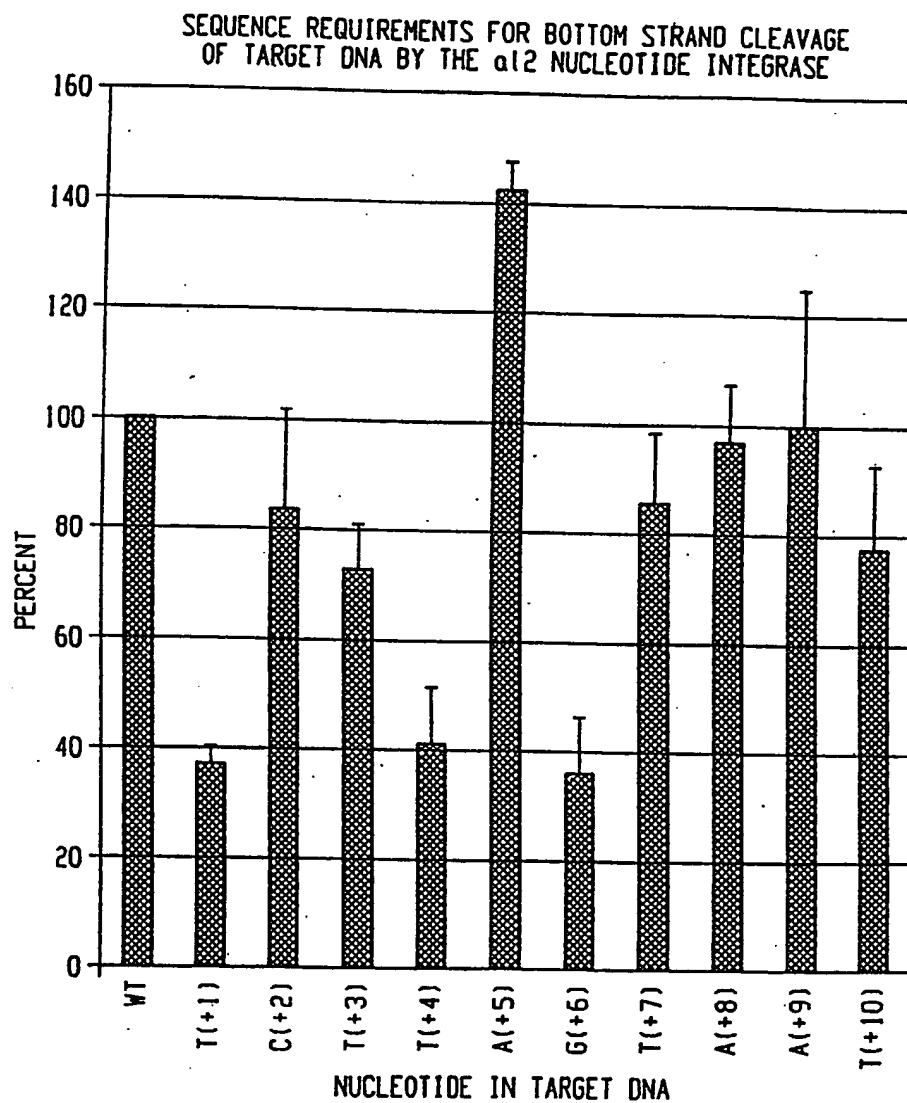


Fig. 4

8/24

*Fig. 5*

9/24

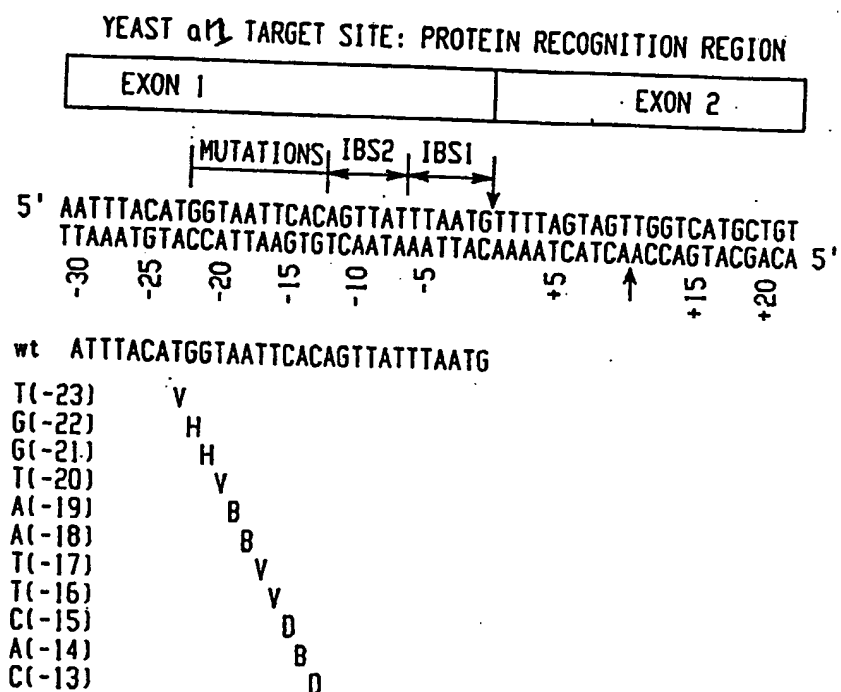
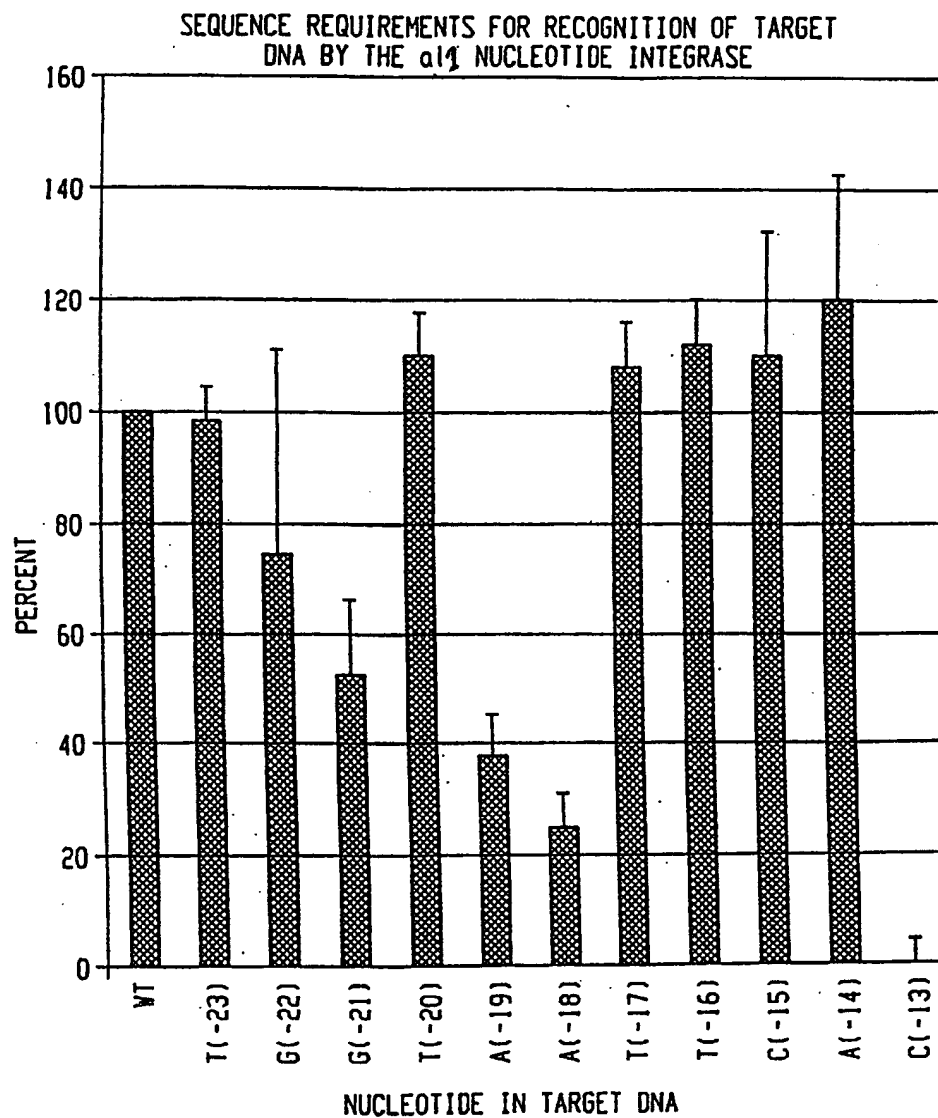


Fig. 6

*Fig. 7*

LACTOCOCCUS ItrA TARGET SITE: PROTEIN RECOGNITION REGION

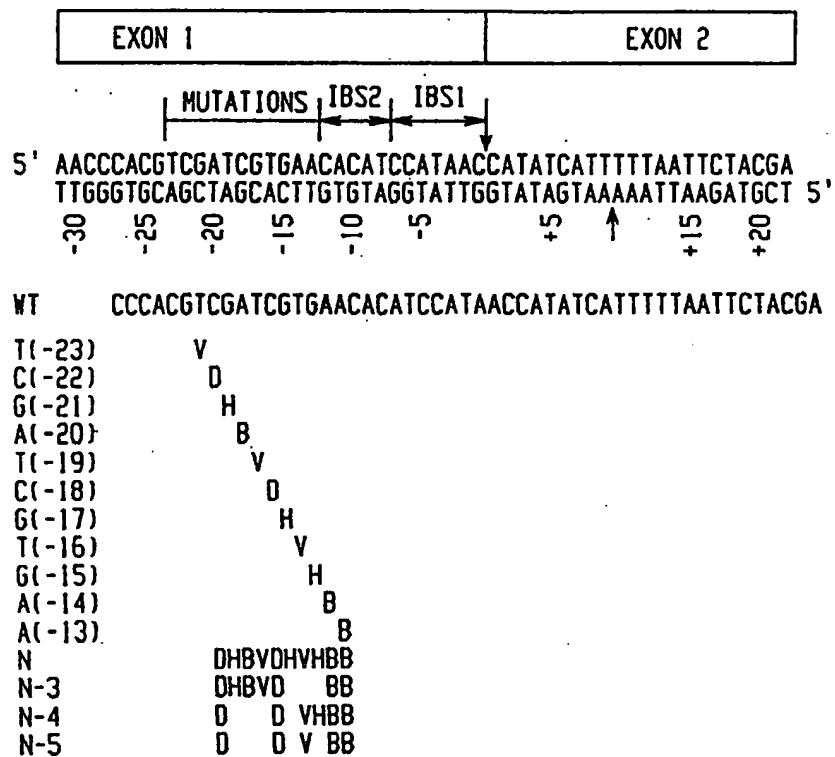
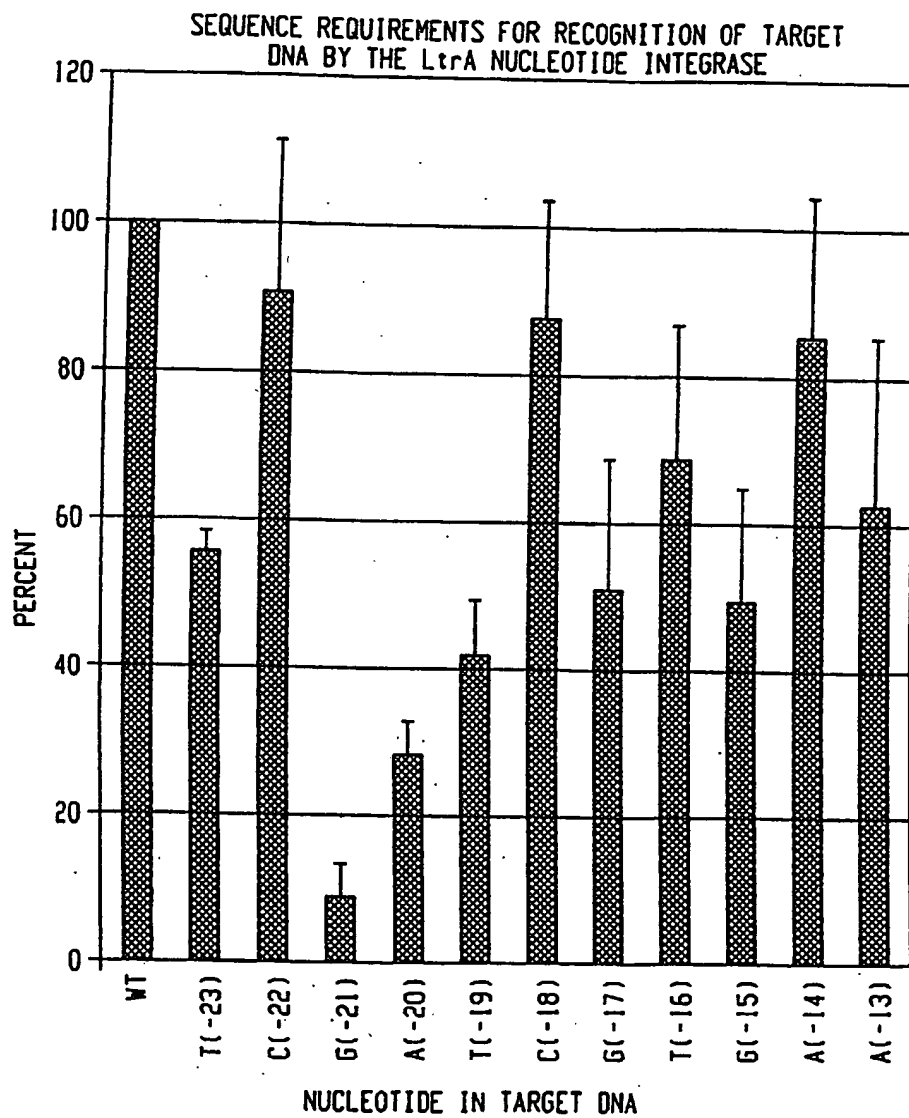


Fig. 8

*Fig. 9*

10 20 30 40 50 60
AAGCTTAGAGAAAAATAATGCGGTGCTTGGTCATCACCTCATCCAATCATTCTCTCTGA
TTCGAATCTCTTTTATTACGCCACGAACCAAGTAGTGGAGTAGGTTAGTAAAAGAGGACT

70 80 90 100 110 120
TGACAATCTAACTCCTGAACAAATTCATGAAATAGGTCGTCAAACCATATTAGAATTTAC
ACTGTTAGATTGAGGACTTGTTTAAGTACTTTATCCAGCAGTTTGGTATAATCTTAAATG LtrB. EI

130 140 150 160 170 180
AGGTGGCGAATATGAATTTGTGATTGCAACCCACGTCGATCGTGAACACATCCATAACGT
TCCACCGCTTATACCTAAACACTAACGTTGGGTGCAGCTAGCACTTGTGTAGGTATTGCA LtrBI

190 200 210 220 230 240
GCGCCCAGATAGGGTGTAAAGTCAAGTAGTTTAAAGGTACTACTCTGTAAGATAACACAGA
CGCGGGTCTATCCACAATTCAGTTCATCAAATTCATGATGAGACATTCTATTGTGTCT

250 260 270 280 290 300
AAACAGCCAACCTAACCGAAAAGCGAAAAGCTGATACGGGAACAGAGCACGGTTGGAAAAGC
TTTGTGGTTGGATTGGCTTTTCGCTTCGACTATGCCCTTGTCTCGTGCCAACCTTTCG

310 320 330 340 350 360
GATGAGTTACCTAAAGACAATCGGGTACGACTGAGTCGCAATGTTAATCAGATATAAGGT
CTACTCAATGGATTCTGTAGCCCATGCTGACTCAGCGTTACAATTAGTCTATATTCCA

370 380 390 400 EBS2 410 420
ATAAGTTGTGTTTACTGAACGCAAGTTTCTAATTTTCGGTTATGTGTGATAGAGGAAAGT
TATTCACACAAATGACTTGCCTTCAAAGATTAAAGCCAATACACAGCTATCTCCTTTCA

430 440 450 460 EBS1 470 480
GTCTGAAACCTCTAGTACAAAGAAAGGTAAGTTATGGTTGTGGACTTATCTGTTATCACC
CAGACTTTGGAGATCATGTTTCTTTCCATTCAATACCAACACCTGAATAGACAATAGTGG

490 500 510 520 530 540
ACATTTGTACAATCTGTAGGAGAACCTATGGGAACGAAACGAAAGCGATGCCGAGAATCT
TGTAACATGTTAGACATCCTCTTGGATACCTTGCTTTGCTTTCGCTACGGCTCTTAGA

550 560 570 580 590 600
GAATTTACCAAGACTTAACACTAACTGGGGATACCTAAACAAGAATGCCTAATAGAAAAG
CTTAAATGGTTCTGAATTGTGATTGACCCCTATGGGATTGTCTTACGGATTATCTTTC

MATCH TO FIG. 10B

Fig. 10A

MATCH TO FIG. 10A

610 620 630 640 650 660
GAGGAAAAAGGCTATAGCACTAGAGCTTGAAAATCTTGCAAGGGTACGGAGTACTCGTAG
CTCCTTTTTCCGATATCGTGATCTCGAACTTTAGAACGTTCCCATGCCTCATGAGCATC

670 680 690 700 710 720
TAGTCTGAGAAGGGTAACGCCCTTTACATGGCAAAGGGGTACAGTTATTGTGTACTAAAA
ATCAGACTCTTCCCATTCGGGAAATGTACCGTTTCCCATGTCAATAACACATGATTTT

730 740 750 760 770 780
TTAAAAATTGATTAGGGAGGAAAACCTCAAAATGAAACCAACAATGGCAATTTTAGAAAG
AATTTTAACTAATCCCTCCTTTTGGAGTTTACTTTGGTTGTTACCGTTAAATCTTTC

790 800 810 820 830 840
AATCAGTAAAAATTCACAAGAAAATATAGACGAAGTTTTTACAAGACTTTATCGTTATCT
TTAGTCATTTTAAAGTGTCTTTTATATCTGCTTCAAAATGTTCTGAAATAGCAATAGA

850 860 870 880 890 900
TTTACGTCCAGATATTTATTACGTGGCGTATCAAAATTTATATTCCAATAAAGGAGCTTC
AAATGCAGGTCTATAAATAATGCACCGCATAGTTTAAATATAAGGTTATTTCTCGAAG

910 920 930 940 950 960
CACAAAAGGAATATTAGATGATACAGCGGATGGCTTTAGTGAAGAAAAATAAAAAAGAT
GTGTTTTCTTATAATCTACTATGTGCGCTACCGAAATCACTTCTTTTTATTTTTCTA

970 980 990 1000 1010 1020
TATTCAATCTTTAAAGACGGAACCTTACTATCCTCAACCTGTACGAAGAATGTATATTGC
ATAAGTTAGAAATTTCTGCGTTGAATGATAGGAGTTGGACATGCTTCTTACATATAACG

1030 1040 1050 1060 1070 1080
AAAAAGAATTCTAAAAAGATGAGACCTTTAGGAATCCAACCTTTCACAGATAAATTGAT
TTTTTCTTAAGATTTTCTACTCTGGAAATCCTTAAGGTTGAAAGTGTCTATTTAACTA

1090 1100 1110 1120 1130 1140
CCAAGAAGCTGTGAGAATAATTCTTGAATCTATCTATGAACCGGTATTGGAAGATGTGTC
GGTCTTCGACACTCTTATTAAGAACTTAGATAGATACTTGGCCATAAGCTTCTACACAG

1150 1160 1170 1180 1190 1200
TCACGGTTTTAGACCTCAACGAAGCTGTACACAGCTTTGAAAACAATCAAAAGAGAGTT
AGTGCCAAAATCTGGAGTGGCTTCGACAGTGTGCGAACTTTTGTAGTTTTCTCTCAA

MATCH TO FIG. 10C

Fig. 10B

MATCH TO FIG. 10B

1210 1220 1230 1240 1250 1260
TGGCGGCGCAAGATGGTTTGTGGAGGGAGATATAAAGGCTGCTTCGATAATATAGACCA
ACCGCCGCGTCTACCAAACACCTCCCTCTATATTTCCGACGAAGCTATTATATCTGGT

1270 1280 1290 1300 1310 1320
CGTTACACTCATTGGACTCATCAATCTTAAATCAAAGATATGAAAATGAGCCAATTGAT
GCAATGTGAGTAACCTGAGTAGTTAGAATTTAGTTTCTATACTTTTACTCGGTTAACTA

1330 1340 1350 1360 1370 1380
TTATAAATTTCTAAAAGCAGGTTATCTGGAAAACCTGGCAGTATCACAAAACCTACAGCGG
AATATTTAAAGATTTTCGTCCAATAGACCTTTTGACCGTCATAGTGTGTTGAATGTCGCC

1390 1400 1410 1420 1430 1440
AACACCTCAAGGTGGAATTCTATCTCCTCTTTTGGCCAACATCTATCTTCATGAATTGGA
TTGTGGAGTTCACCTTAAGATAGAGGAGAAAACCGGTTGTAGATAGAAGTACTTAACCT

1450 1460 1470 1480 1490 1500
TAAGTTTGTGTTTACAACCTCAAATGAAGTTTGACCGAGAAAGTCCAGAAAGAATAACACC
ATTCAAACAAAATGTTGAGTTTACTTCAAACCTGGCTCTTTCAGGTCTTCTTATTGTGG

1510 1520 1530 1540 1550 1560
TGAATATCGGGAACCTCACAAATGAGATAAAAAGAATTTCTCACCCTCTCAAGAAAGTTGGA
ACTTATAGCCCTTGAAGTGTACTCTATTTTCTTAAAGAGTGGCAGAGTCTTCAACCT

1570 1580 1590 1600 1610 1620
GGGTGAAGAAAAAGCTAAAGTTCTTTTAGAATATCAAGAAAAACGTAAAAGATTACCCAC
CCCACTTCTTTTTCGATTTCAAGAAATCTTATAGTTCTTTTGCATTTTCTAATGGGTG

1630 1640 1650 1660 1670 1680
ACTCCCCTGTACCTCACAGACAAATAAAGTATTGAAATACGTCCGGTATGCGGACGACTT
TGAGGGGACATGGAGTGTCTGTTTATTTTCACTTTATGCAGGCCATACGCCTGCTGAA

1690 1700 1710 1720 1730 1740
CATTATCTCTGTTAAAGGAAGCAAAGAGGACTGTCAATGGATAAAAGAACAATTAACCT
GTAATAGAGACAATTTCCCTTCGTTTCTCCTGACAGTTACCTATTTTCTTGTTAATTTGA

1750 1760 1770 1780 1790 1800
TTTTATTTCATAACAAGCTAAAAATGGAATTGAGTGAAGAAAAACACTCATCACACATAG
AAAATAAGTATTGTTTCGATTTTACCTTAACCTCACTTCTTTTGTGAGTAGTGTGTATC

MATCH TO FIG. 10D

Fig. 10C

MATCH TO FIG. 10C

1810 1820 1830 1840 1850 1860
CAGTCAACCCGCTCGTTTTCTGGGATATGATATACGAGTAAGGAGAAGTGGAACGATAAA
GTCAGTTGGGCGAGCAAAAGACCCTATACTATATGCTCATTCTCTTCACCTTGCTATTT

1870 1880 1890 1900 1910 1920
ACGATCTGGTAAAGTCAAAAAGAGAACTCAATGGGAGTGAGAACTCCTTATTCCTCT
TGCTAGACCATTTCAGTTTTTCTCTTGAGTTACCCTCACATCTTGAGGAATAAGGAGA

1930 1940 1950 1960 1970 1980
TCAAGACAAAATTCGTCAATTTATTTTGACAAGAAAATAGCTATCCAAAAGAAAGATAG
AGTTCTGTTTTAAGCAGTTAAATAAAACTGTTCTTTATCGATAGGTTTTCTTCTATC

1990 2000 2010 2020 2030 2040
CTCATGGTTTCCAGTTCACAGGAAATATCTTATTCGTTCAACAGACTTAGAAATCATCAC
GAGTACCAAAGGTCAAGTGTCTTTATAGAATAAGCAAGTTGTCTGAATCTTTAGTAGTG

2050 2060 2070 2080 2090 2100
AATTTATAATTCTGAATTAAGAGGGATTTGTAATTACTACGGTCTAGCAAGTAATTTTAA
TTAAATATTAAGACTTAATTCTCCCTAACATTAATGATGCCAGATCGTTCATTAAAAAT

2110 2120 2130 2140 2150 2160
CCAGCTCAATTATTTTGCTTATCTTATGGAATACAGCTGTCTAAAAACGATAGCCTCCAA
GGTCGAGTTAATAAACGAATAGAATACCTTATGTCGACAGATTTTGTCTATCGGAGGTT

2170 2180 2190 2200 2210 2220
ACATAAGGGAACACTTTCAAAAACCATTTCATGTTTAAAGATGGAAGTGGTTCGTGGGG
TGATTCCCTTGTAAGTTTTTGGTAAAGGTACAAATTTCTACCTCACCAAGCACCCC

2230 2240 2250 2260 2270 2280
CATCCCGTATGAGATAAAGCAAGGTAAGCAGCGCCGTTATTTTGCAAATTTTAGTGAATG
GTAGGGCATACTCTATTTCTGTTCCATTCTGTCGCGCAATAAACGTTTAAATCACTTAC

2290 2300 2310 2320 2330 2340
TAAATCCCTTATCAATTTACGGATGAGATAAGTCAAGCTCCTGTATTGTATGGCTATGC
ATTTAGGGGAATAGTTAAATGCCTACTCTATTCAGTTTCGAGGACATAACATACCGATACG

2350 2360 2370 2380 2390 2400
CCGGAATACTCTTGAAAACAGGTTAAAGCTAAATGTTGTGAATTATGTGGAACATCTGA
GGCCTTATGAGAACTTTTGCCAATTTTCGATTACAACACTTAATAACCTTGAGACT

MATCH TO FIG. 10E

Fig. 10D

MATCH TO FIG. 10D

2410	2420	2430	2440	2450	2460	
TGAAAATACCTTCCTATGAAATTCACCATGTCAATAAGGTCAAAAATCTTAAAGGCAAAGA						
ACTTTTATGAAGGATACTTTAAGTGGTACAGTTATTCAGTTTTTAGAATTTCCGTTTCT						
2470	2480	2490	2500	2510	2520	
AAAATGGGAAATGGCAATGATAGCGAAACAACGTAAACTCTTGTGTATGCTTTTCATTG						
TTTACCCTTTACCGTTACTATCGCTTTGTTGCATTTTGAGAACAACATACGAAAGTAAC						
2530	2540	2550	2560	2570	2580	
TCATCGTCACGTGATTTCATAAACACAAGTGAATTTTACQAACGAACAATAACAGAGCCG						
AGTAGCAGTGCACATAAGTATTTGTGTTCACTTAAAAATGCTTGCTTGTATTGTCTCGGC						
2590	2600	2610	2620	2630	2640	
TATACTCCGAGAGGGGTACGTACGGTTCCCGAAGAGGGTGGTGCAAACCAAGTCACAGTAA						LtrB1
ATATGAGGCTCTCCCATGCATGCCAAGGGCTTCTCCACCACGTTTGGTCAGTGTCAAT						
2650	2660	2670	2680	2690	2700	
TGTGAACAAGGCGGTACCTCCCTACTTCACCATATCATTTTTTAATTCTACGAATCTTTAT						LtrB. E2
ACACTTGTTCGCCCATGGAGGGATGAAGTGGTATAGTAAAAATTAAGATGCTTAGAAATA						
2710	2720	2730	2740	2750	2760	
ACTGGCAAACAATTTGACTGGAAAGTCATTCTAAAGAGAAAACAAAAGCGGCAAAGCT						
TGACCGTTTGTTAACTGACCTTTCAGTAAGGATTTCTTTTGTGTTTTTCGCCGTTTCGA						

T
A

Fig. 10E

10	20	30	40	50	60
MKPTMAILERISKNSQENIDEVFTRLYRYLLRFDIYVAYONLYSNKGASTAGILDDTAD					
70	80	90	100	110	120
GFSEEEKIKKIIQSLKDOTYYYPQVRRMTIAKNSKMRPLGIPTFTDKLIQEAVRIILES					
130	140	150	160	170	180
IYBPVVFEDVSHGFRPQRSCHTALRTIKREFGGARWFEVDIKGCFDNIDHVTLIGLINLK					
190	200	210	220	230	240
IKDKMSQLIYKPLKAGYLENWQYHKTYSGTPQGGILSPLLANTYLHELDKPFVLQKMKF					
250	260	270	280	290	300
DRESPERITPEYRELHNEIKRISHRLKKEGEKAKVLLEYQEKRKRLPTLPCTSQTNKV					
310	320	330	340	350	360
LKYVRYADDFIISVKGSKEDCQWIKQLKLFTHNKLKMLSEKTLITHSSQPARFLGYD					
370	380	390	400	410	420
IRVRRSGTIKRSQKVKKRTINGSVELLIPLODKIRQFIFDKKIAIQKKDSSWFFVHRKYL					
430	440	450	460	470	480
IRSTDLBIITIIYNSBLRGICNYYGLASNFWQLNYFATLMEYSCLKTIASKHKGTLSKTIS					
490	500	510	520	530	540
MFKDQSGSWGIPYBIKQKQRRYFANFSECKSPYQFTDEISQAPVLYGYARNTLENRLKA					
550	560	570	580	590	600
KCCELCOTSDENTSYEIHHVNKVNKLGKEKNEAMIAKQKRTLVCVCFHCHRVHVKHK*					

Fig. 11

Lactococcus target DNA: mutant oligos for top strand: MUTATIONS ARE UNDERLINED

		Pvu I	E1		E2		EcoR I	
			↓					
		CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTAAATTCACGAATCTTTATACTGGgaattccg						72
		GGGTGCAGCTAGCACTTGTGTAGGTATTGGTATAGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc						
WT	CCCACGTCGATCGTGAACACATCCATA							
G-24H	CCCACGTCGATCGTGAACACATCCATA							
T-23V	CCCACGTCGATCGTGAACACATCCATA							
C-22D	CCCACGTCGATCGTGAACACATCCATA							
G-21H	CCCACGTCGATCGTGAACACATCCATA							
A-20B	CCCACGTCGATCGTGAACACATCCATA							
T-19V	CCCACGTCGATCGTGAACACATCCATA							
C-18D	CCCACGTCGATCGTGAACACATCCATA							
G-17H	CCCACGTCGATCGTGAACACATCCATA							
T-16V	CCCACGTCGATCGTGAACACATCCATA							
G-15H	CCCACGTCGATCGTGAACACATCCATA							
A-14B	CCCACGTCGATCGTGAACACATCCATA							
A-13B	CCCACGTCGATCGTGAACACATCCATA							
LLN	CCCACGTCGATCGTGAACACATCCATA							
LLN-2	CCCACGTCGATCGTGAACACATCCATA							
LLN-3A	CCCACGTCGATCGTGAACACATCCATA							
LLN-4	CCCACGTCGATCGTGAACACATCCATA							
LLN-5	CCCACGTCGATCGTGAACACATCCATA							
LLN-3B	CCCACGTCGATCGTGAACACATCCATA							
GC1	CCCACGTCGATCGTGAACACATCCATA							
GC2	CCCACGTCGATCGTGAACACATCCATA							
WT2	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-13Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C-12R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-11Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C-10R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-9Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
T-8R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C-7R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C-6R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-5Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
T-4R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-3Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
A-2Y	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C-1R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
C+1R	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
cIBS1IBS2	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							
c9875321	CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTT							

Fig. 12

Lactococcus target DNA: mutant oligos for top strand: MUTATIONS ARE UNDERLINED

	Pvu I	EcoR I	
			CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTTAATTCTACGAATCTTTATACTGGgaattccg 72
			GGGTGCAGCTAGCACTTGTGTAGGTATTGGTATAGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc
L753			CCCACGTCGATCGTGAACACAT <u>TCCTT</u> ACCATATCATTTTT
C-7T			CCCACGTCGATCGTGAACACAT <u>TC</u> CATAACCATATCATTTTT
C-7A			CCCACGTCGATCGTGAACACAT <u>TC</u> CATAACCATATCATTTTT
C-7G			CCCACGTCGATCGTGAACACAT <u>TC</u> CATAACCATATCATTTTT
A-5T			CCCACGTCGATCGTGAACACATCCCTAACCATATCATTTTT
A-5C			CCCACGTCGATCGTGAACACATCCCTAACCATATCATTTTT
A-5G			CCCACGTCGATCGTGAACACATCCCTAACCATATCATTTTT
A-3T			CCCACGTCGATCGTGAACACATCCATTACCATATCATTTTT
A-3C			CCCACGTCGATCGTGAACACATCCATTACCATATCATTTTT
A-3G			CCCACGTCGATCGTGAACACATCCATTACCATATCATTTTT
C-12G			CCCACGTCGATCGTGAAGACATCCATAACCATATCATTTTT
T-11A			CCCACGTCGATCGTGAAGTCCATCCATAACCATATCATTTTT
C-10G			CCCACGTCGATCGTGAAGATCCATAACCATATCATTTTT
T-4G			CCCACGTCGATCGTGAACACATCCAGAACCATATCATTTTT
C+1G			CCCACGTCGATCGTGAACACATCCATAACGATATCATTTTTTAA
A+2T			CCCACGTCGATCGTGAACACATCCATAACCTTATCATTTTTTAA
T+3A			CCCACGTCGATCGTGAACACATCCATAACCAATCATTTTTTAA
T+3G			CCCACGTCGATCGTGAACACATCCATAACCAATCATTTTTTAA
A+4T			CCCACGTCGATCGTGAACACATCCATAACCAATTCATTTTTTAA
T+5G			CCCACGTCGATCGTGAACACATCCATAACCATAGCATTTTTTAA
TopI			CCCACGTCGATCGTGAACACATCCATAACCTGGCATTTTTTAA

Lactococcus target DNA: mutant oligos for bottom strand

	Pvu I	EcoR I	
			CCCACGTCGATCGTGAACACATCCATAACCATATCATTTTTAATTCTACGAATCTTTATACTGGgaattccg 72
			GGGTGCAGCTAGCACTTGTGTAGGTATTGGTATAGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc

MUTATIONS ARE UNDERLINED

LLBOT	GTGTAGGTATTGGTATAGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc
LLBOT2	TATAGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc
LLBOT3	AGTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc
BotI	GTGTAGGTATTGGAC <u>CG</u> GTAAAAATTAAGATGCTTAGAAATATGACCcttaaggc
LLJ' compl	GTGTAGGTATTGGATATCATTTTTTAACTTAGAAAGAAAAAGACCCTTAAGGC

KEY TO MUTATIONS:

B: C or G or T
 D: A or G or T
 H: A or C or T
 Y: A or C or G
 R: A or G
 X: C or T

Fig. 12 (con't)

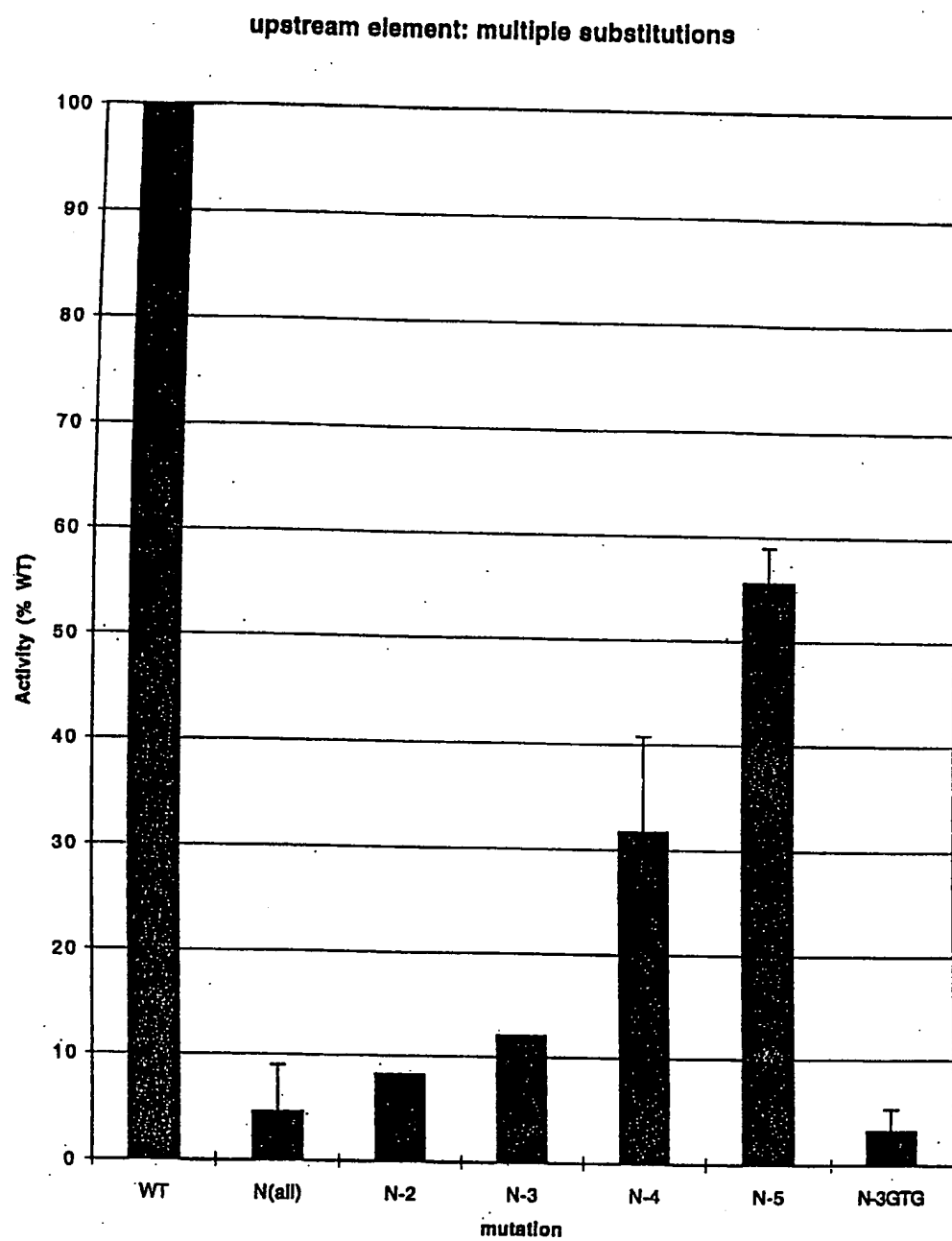


Fig. 13

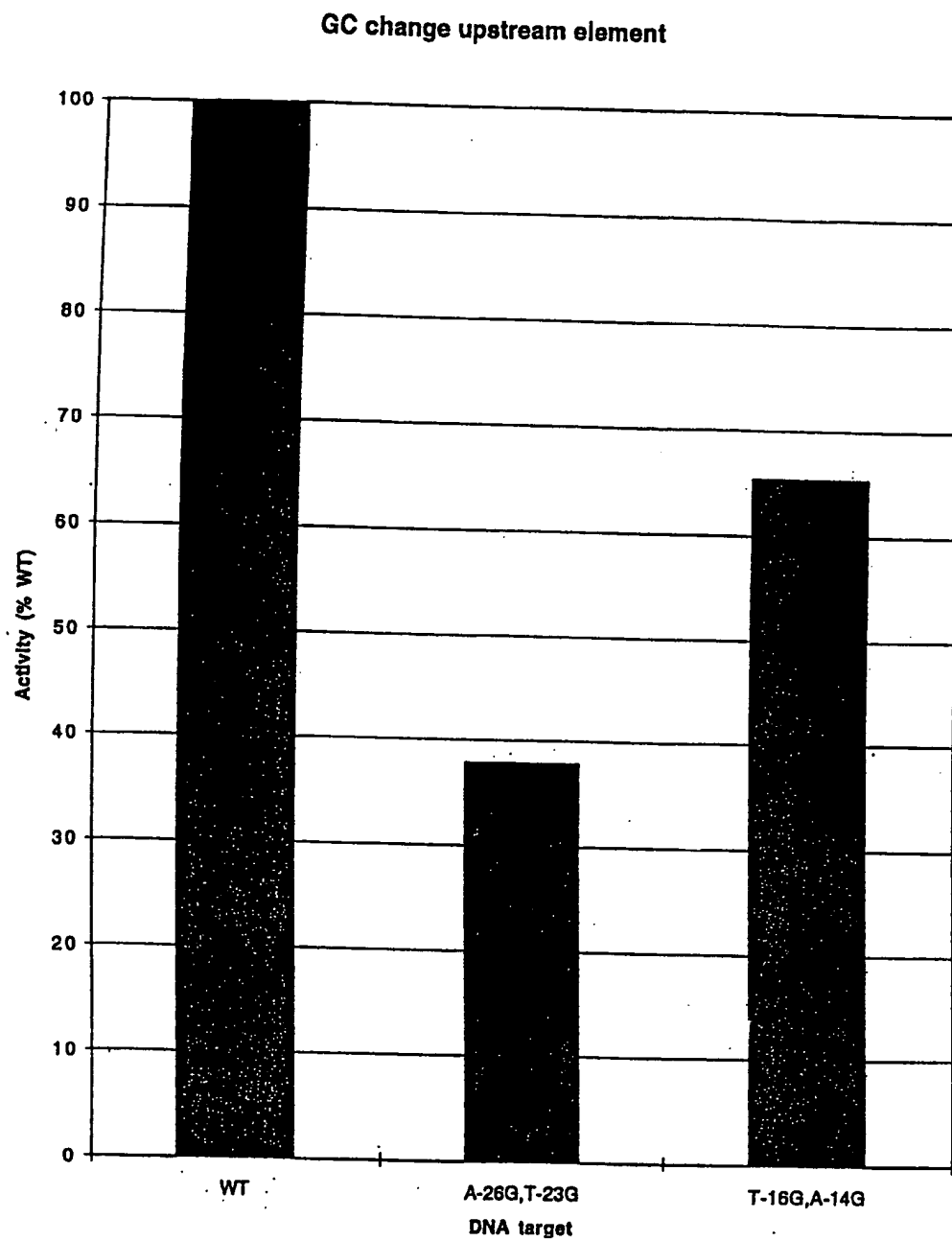


Fig. 14

Summary: LtrA target mutagenesis
IBS1 and IBS2, δ - δ'

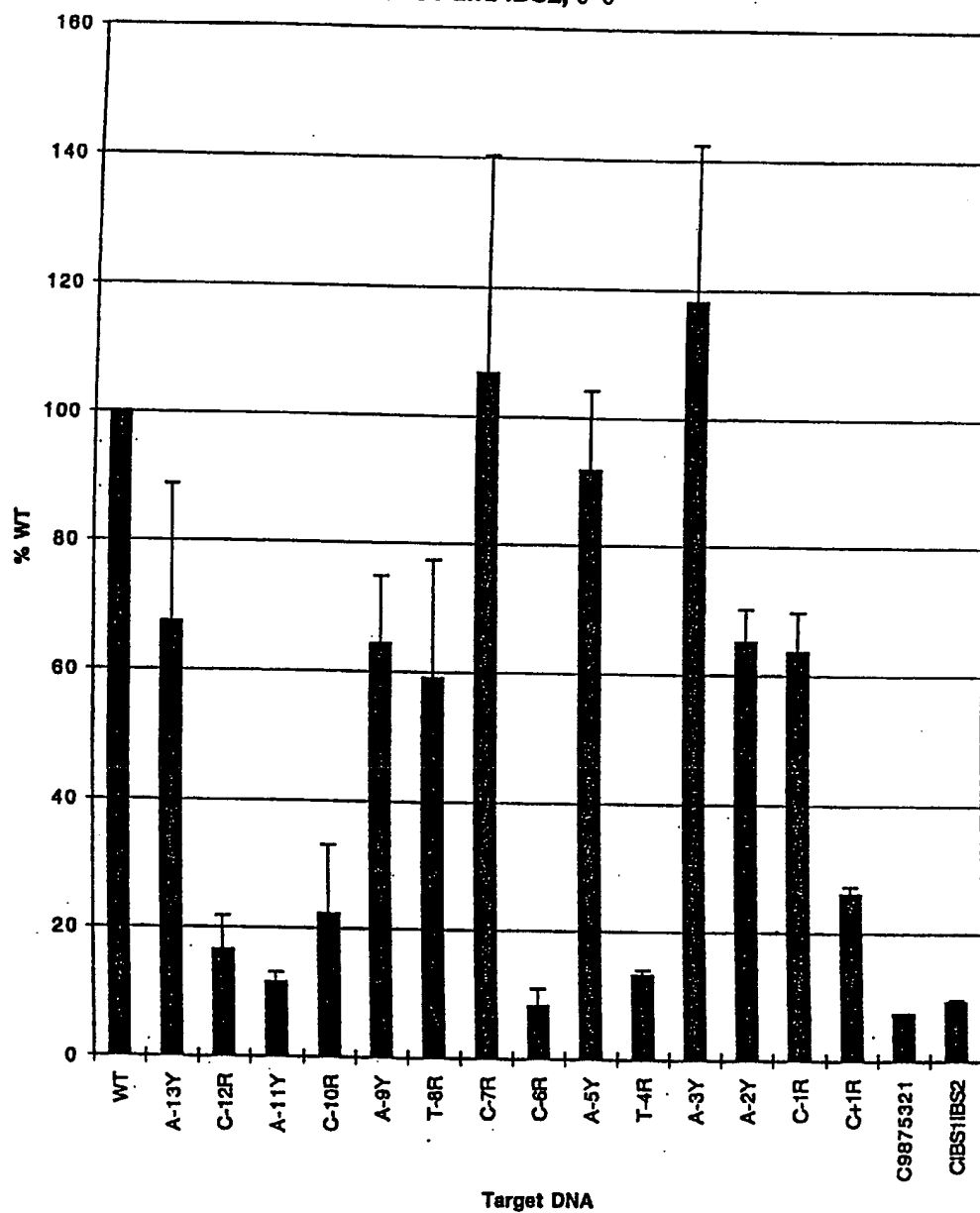


Fig. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04049

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34

US CL :435/6, 91.31, 91.1, 91.51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.31, 91.1, 91.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, CAPLUS, WPIDS, JAPIO

search terms: nucleotide integrase, single stranded DNA, double stranded, 11.ltrb

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZIMMERLY, S. et al. Group II intron mobility occurs by Target DNA- Primed Reverse Transcription. Cell. 25 August 1995, Vol. 82, pages 545-554, see entire document.	1-15
Y	YANG, J. et al. Efficient integration of an intron RNA into double-stranded DNA by reverse splicing. Nature. 23 May 1996, Vol. 381, pages 332-335, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 JUNE 1999

Date of mailing of the international search report

08 JUL 1999

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